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MUC5B glycosylation in human saliva reflects blood group and secretor status

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This study aimed to characterize human salivary glycoforms and the natural glycosylation variation of the major ABO blood group bearing high molecular weight glycoprotein fraction MG1, which mainly consists of MUC5B mucin. Reduced and alkylated mucins from individuals of blood group A, B, and O were purified by sodium dodecyl sulfate-agarose/polyacrylamide composite gel electrophoresis (SDS–AgPAGE), blotted to polyvinylidene fluoride (PVDF) membranes, and visualized with alcian blue. O-linked oligosaccharides were released from MUC5B glycoform bands by reductive β-elimination and analyzed by liquid chromatography (LC) electrospray ion trap mass spectrometry (MS). Slow electrophoretically migrating MUC5B components (sm) were found to be dominated by neutral oligosaccharides, and fast-migrating (fm) components were dominated by sulfated oligosaccharides. ABO blood group-specific sequences were found on all glycoforms, and novel oligosaccharides containing blood group A and B type sequences were sequenced. This is the first molecular description of the influence of the blood group ABO system on salivary MUC5B oligosaccharides. Expanding these results from the three A, B, and O individuals into larger population (29 individuals), we found oligosaccharide sequences corresponding to the blood group of the donor on MUC5B from 23 individuals. The remaining six individuals were characterized by a high degree of sialylation. These individuals were assigned as nonsecretors, whereas blood group-expressing individuals were assigned as secretors. Western blot assays with antibodies confirmed increased expression of Sialyl Lewis a (Si-Le^a) in the nonsecretors. Our results highlight that salivary MUC5B consists of glycoforms with distinct glycosylation that vary extensively between individuals and that some of this variation is owing to blood group and secretor status.

Key words: glycoforms/mass spectrometry/MUC5B/saliva

Introduction

Salivary secretions are essential for the protection and lubrication of the oral surfaces in the mouth. The high turnover rate of the saliva promotes clearance of trapped bacteria, and the individual salivary protein components regulate the oral flora by providing an antimicrobial activity. Mucins are major protein components in saliva. Mucins are multimerising macromolecules with an extensive O-linked glycosylation (50–80 wt %) of Ser/Thr/Pro rich domains (Moniaux *et al*., 2001). Mucin oligosaccharides contribute to the viscous and adhesive properties of saliva, as well as providing interaction sites for bacteria and for leukocyte interactions (reviewed by Zalewska *et al*., 2000).

Two populations of mucins with distinct features are present in saliva: MG1, a high molecular mass component (M_r >10^6), and MG2 (M_r 1.2–1.5 × 10^5) (van Nieuw Amerongen *et al*., 1995). The major protein component of MG1 is encoded by the *MUC5B* gene (Nielsen *et al*., 1997; Wickström *et al*., 1998; Thornton *et al*., 1999) and is a large, gel-forming, multimerising mucin, produced by mucosal cells (Nielsen *et al*., 1996). MG2 consists of a small, nonmultimerising protein encoded by *MUC7* (Bobek *et al*., 1993) and is secreted by serous cells (Nielsen *et al*., 1996). Both mucin populations appear to make up part of the salivary pellicle that coats the tooth surface and interacts with different species of bacteria (Murray *et al*., 1992). The two mucin populations both appear as glycoforms (Ramasubbu *et al*., 1991; Bolscher *et al*., 1995) and carry different glycosylation, with MUC5B containing on average longer and more diverse oligosaccharides than MUC7 (Prakobphol *et al*., 1998; Thomsson *et al*., 2002). A previous study performed on the high molecular weight fraction of saliva suggests that MUC5B is the main mucin carrier of blood group antigens (Prakobphol *et al*., 1993). Different α1,2fucosyltransferase gene products in blood and body secretions are responsible for the production of the main blood group antigen saccharide precursor, the H epitope (Fucα1-2Gal) (reviewed by Henry *et al*., 1995). The Secretor (Se) fucosyltransferase is expressed in salivary glands, and individuals screened for ABH determinants in their saliva using immunological methods have been named "secretors" or "nonsecretors" depending on the presence or absence of these epitopes. In the Caucasian population, the distribution between secretors and nonsecretors is ~4:1.

The aim of this study was to characterize MUC5B glycoforms and to investigate to what extent salivary MUC5B glycosylation varies between individuals. This was performed by using an approach described in recent works (Schulz *et al*., 2002a,b), involving gel electrophoresis of reduced and alkylated saliva, electroblotting of protein components to polyvinylidene fluoride (PVDF) membrane, release of oligosaccharides, and analysis with liquid chromatography–mass spectrometry (LC-MS) and LC-tandem MS (LC-MS/MS).

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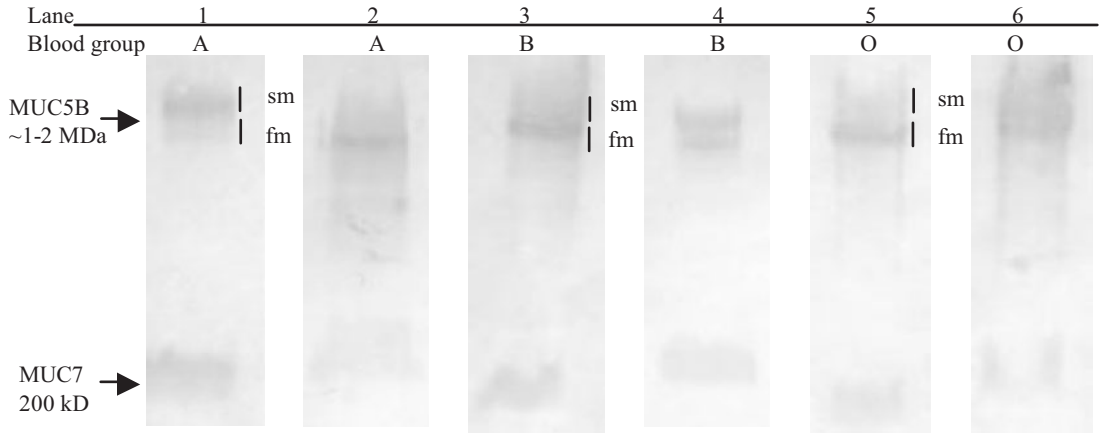

Fig. 1. Mucin components from secretors. Saliva was reduced and alkylated, separated by sodium dodecyl sulfate-agarose/polyacrylamide composite gel electrophoresis (SDS–AgPAGE), blotted to polyvinylidene fluoride (PVDF) membrane, and stained with alcian blue. MUC5B glycoforms were cut out as shown. Each lane is annotated with the ABO blood group of the donor. (fm = fast-migrating MUC5B components, sm = slow-migrating MUC5B components).

Results

Gel electrophoresis of salivary MUC5B and screening of oligosaccharides from blood group A, B, and O individuals

Fresh saliva from six individuals was reduced, alkylated, separated with 1 D sodium dodecyl sulfate-agarose/polyacrylamide composite gel electrophoresis (SDS–AgPAGE) gradient gels, blotted onto PVDF membranes, and stained with alcian blue, which stains acidic oligosaccharides. MUC5B appeared as one or more bands at 1–2 MDa apparent molecular weight (Figure 1), and its position in the gel varied somewhat. However, the differences in the migration were not correlated with the ABO phenotype of the individuals. O-linked oligosaccharides were released by β-elimination from the slow- (sm) and fast-migrating (fm) MUC5B components from one blood group A, one blood group B, and one blood group O individual according to Figure 1. The oligosaccharides were analyzed with LC-MS and MS/MS. The total ion chromatograms from the six analyses are shown in Figure 2, where major peaks are annotated with pseudomolecular $[M–xH]^{x–}$ and oligosaccharide composition. The sm MUC5B from individuals with different ABO blood groups (Figure 2: upper panels) contained glycoforms dominated by neutral oligosaccharides, whereas fm MUC5B glycoforms (Figure 2: lower panels) were dominated by sulfated oligosaccharides. All major peaks were fucosylated.

MUC5B neutral glycoforms from blood group A, B, and O individuals

Further analysis of the glycosylation of MUC5B from three individuals (from Figure 1) representing each of the three blood groups were performed to elucidate blood group-specific variations. The major oligosaccharide components from the sm MUC5B (Figure 1: bands labeled sm; Figure 2: upper panels) were shown to differ between the three samples. The composition of detected molecular ions together with sequences are listed in Table I. The major differences in the sm MUC5B from different individuals were owing to an extensive expression of neutral oligosaccharides corresponding to the individuals' ABO blood type. The O individual expressed blood group H epitopes on the oligosaccharides, whereas the blood group A and blood group B individuals in addition to H epitopes expressed blood group A and blood group B antigens on MUC5B, respectively. The molecular basis for the blood group-related epitopes and the corresponding sequences detected by MS are summarized in Table II.

MUC5B sulfated glycoforms from blood group A, B, and O individuals

The oligosaccharide profiles obtained of the fm "sulfated" glycoforms from the three different ABO individuals (Figure 1: bands labeled fm, Figure 2: lower panels) consisted mainly of monosulfated oligosaccharides, though disulfated and sialic acid-containing oligosaccharides were also detected. The compositions of identified molecular ions are listed together with elucidated sequences in Table I.

MS/MS and MS^3 were used to fully sequence the dominating sulfated oligosaccharides (such as the $[M–H]^–$ ion of *m/z* 1340 and *m/z* 1381) and to assign the sulfate to a monosaccharide unit. Figure 3 shows the MS/MS spectrum of *m/z* 1340 (Figure 3A) and MS^3 spectrum of the daughter ion at *m/z* 667 (Figure 3B) from the blood group B individual. Figure 3A shows the series of Y_i fragments and one $B_{2\alpha}$ fragment, from which part of the two branches can be elucidated: one branch carrying a blood group B sequence linked to a core *N*-acetylhexosamine (HexNAc) residue and the second branch consisting of a Hexose (Hex) and a HexNAc unit with a sulfate group attached to either sugar. The exact arrangement of the second branch was determined by using MS^3 of the Y fragment at *m/z* 667 (Figure 3B), giving an intense fragment at *m/z* 241, diagnostic for sulfate linked to a Hex residue. The lack of a fragment indicating the loss of a terminal HexNAc residue at *m/z* 1137 indicates that the sequence contains a core 4 arrangement, where the full sequence is

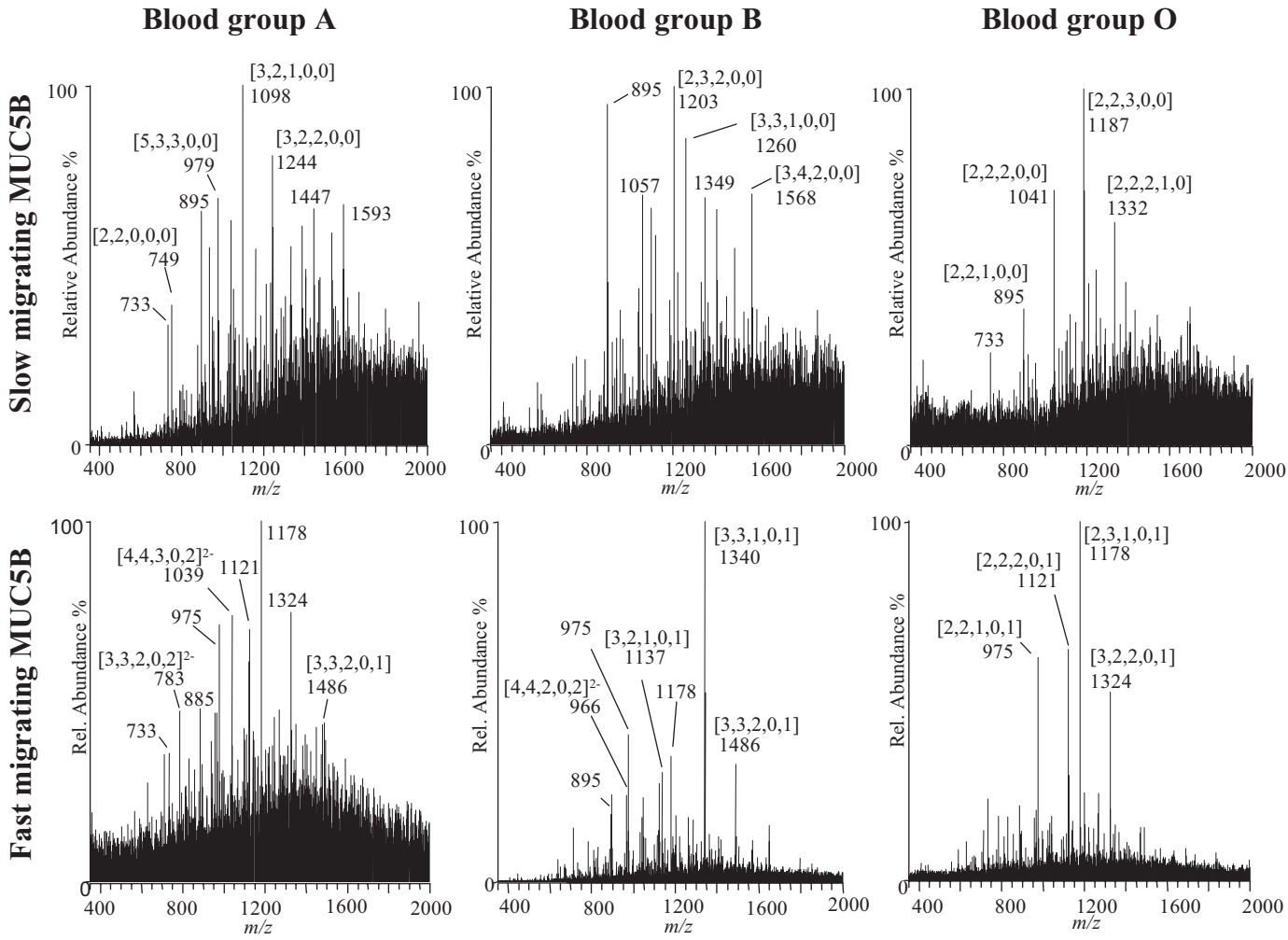

Fig. 2. Total ion mass spectra from liquid chromatography–mass spectrometry (LC-MS) of O-linked oligosaccharides released from salivary MG1 (MUC5B) glycoforms, separated by sodium dodecyl sulfate-agarose/polyacrylamide composite gel electrophoresis (SDS–AgPAGE), and blotted to polyvinylidene fluoride (PVDF) membrane. Upper panels: slow-migrating (sm) MUC5B components from A, B, and O secretors. Lower panel: fast-migrating (fm) MUC5B components from A, B, and O. The analyzed components are indicated in Figure 1. Peaks are labeled with *m/z*, charge state if *z* is >1, and monosaccharide composition [*N*-acetylhexosamine (HexNAc), Hexose (Hex), fucose (Fuc), *N*-acetylneuraminic acid (NeuAc), and sulfate]. For simplicity, the terminal *N*-acetylhexosaminitol (HexNAcol) is included as an HexNAc. Only major peaks were assigned. Comprehensive list of detected structures and compositions is found in Table I.

HSO_3-Hex-HexNAc-(Hex-(Fuc-)Hex-HexNAc-)HexNAcol. In addition to the sequence corresponding to *m/z* 1340, two additional sulfated oligosaccharides containing blood group B sequences from the blood group B individual (Figure 2) were identified, one having the sequence Hex-(Fuc-)Hex-(HSO_3–)HexNAc-HexNAcol (*m/z* 975) and the other at *m/z* 1121 with the same sequence containing a Fuc residue linked to the sulfated HexNAc residue.

Using this approach, we have characterized two MUC5B glycoforms derived from each of three individuals with distinct glycosylation (Table I), in part due to the presence of blood group-specific sequences. The sulfation of MUC5B was shown to be linked to both Hex (galactose) and HexNAc (*N*-acetylglucosamine). Because similar oligosaccharide sequences were found in both sm and fm MUC5B glycoforms from the same individual, it can be hypothesized that the faster migration was caused by the addition of sulfate (and increased charge) to neutral oligosaccharides.

ABH blood group sequences and secretor status

Saliva from 29 individuals with known ABO blood group was collected and analyzed as described above. In the initial screening, to correlate blood group with expressed MUC5B oligosaccharides, we used the retention time and MS/MS spectra of the diagnostic structures HexNAc-(Fuc)-Hex-3HexNAcol ($[M–H]^-$ ion of *m/z* 733, blood group A), Hex-(Fuc-)Hex-4HexNAc-HexNAcol ($[M–H]^-$ ion of *m/z* 895, blood group B), Fuc-Hex-3HexNAc-HexNAcol ($[M–H]^-$ ion of *m/z* 733, blood group H), and Fuc-Hex-(Hex-HexNAc-)HexNAcol ($[M–H]^-$ ion of *m/z* 895, blood group H). In 23 of 29 individuals, we identified sequences that corresponded to the blood group of the individual (Table III).

Table I. Oligosaccharides from neutral and sulfated glycoforms from salivary MUC5B of three individuals analyzed with liquid chromatography–mass spectrometry (LC-MS), tandem MS (MS/MS), and MS^3.

			Glycoform and blood group					
			sm			fm		
Molecular ion (observed)	Neutral sequences	Blood group sequences	O	A	B	O	A	B
$[M–H]^-$								
530	Fuc-Hex-3HexNAcol	H		x	x			x
587	Hex-3**(HexNAc-6)**HexNAcol			x	x			x
587	Hex-HexNAc-HexNAcol		x	x	x			x
733	Hex-(Fuc-)HexNAc-HexNAcol	Le					x	
733	Fuc-Hex-3HexNAc-HexNAcol	H	x	x	x	x	x	x
733	HexNAc-(Fuc-)Hex-3HexNAcol	A		x			x	
749	Hex-3**(Hex-HexNAc-6)**HexNAcol		x	x	x	x		
790	HexNAc-**(Hex-4HexNAc-6)**HexNAcol		x	x	x			
895	Hex-(Fuc-)HexNAc-Hex-3HexNAcol	Le		x				
895	Hex-3**(Fuc-Hex-4HexNAc-6)**HexNAcol	H	x	x		x	x	
895	Fuc-Hex-3**(Hex-HexNAc-6)**HexNAcol	H	x	x	x	x	x	x
895	Hex-(Fuc-)Hex-4HexNAc-HexNAcol	B			x			x
936	HexNAc-(Fuc-)Hex-HexNAc-HexNAcol	A		x			x	
936	Fuc-Hex-4HexNAc-(HexNAc-)HexNAcol	H	x	x		x		
952	Hex-HexNAc-(Hex-HexNAc-)HexNAcol		x		x			
1041	Fuc-Hex-3**(Fuc-Hex-4HexNAc-6)**HexNAcol	H	x	x		x		
1041	Hex-3**(Fuc-Hex-(Fuc-)HexNAc-6)**HexNAcol	H, Le	x					
1041	Hex-(Fuc-)HexNAc-(Fuc-)Hex-HexNAcol	Le	x					
1057	Hex-3**(Hex-(Fuc-)Hex-HexNAc-6)**HexNAcol	B			x			
1098	2 HexNAc, 3 Hex, Fuc, HexNAcol			x				
1098	Hex-3**(HexNAc-(Fuc-)Hex-HexNAc-6)**HexNAcol	A		x			x	
1098	HexNAc-**(Hex-(Fuc-)Hex-HexNAc-)**HexNAcol	B			x			x
1098	Fuc-Hex-HexNAc-3**(Hex-HexNAc-6)**HexNAcol	H	x		x	x	x	
1098	Hex-HexNAc-3**(Fuc-Hex-HexNAc-6)**HexNAcol	H		x				
1187	Fuc-Hex-3**(Fuc-Hex-(Fuc-)HexNAc-6)**HexNAcol	H, Le	x					
1203	HexNAc, 3 Hex, 2 Fuc, HexNAcol				x			x
1203	Hex-3**(Hex-(Fuc-)Hex-(Fuc-)HexNAc-6)**HexNAcol	B, Le			x			
1244	Hex-3**(HexNAc-(Fuc-)Hex-(Fuc-)HexNAc-6)**HexNAcol	A, Le		x				
1244	HexNAc-(Fuc-)Hex-3**(Fuc-Hex-4HexNAc-6)**HexNAcol	A, H		x				
1244	Fuc-Hex-4HexNAc-(Fuc-Hex-3HexNAc)HexNAcol	H	x			x	x	

Table I. continued

Molecular ion (observed)	Neutral sequences	Blood group sequences	Glycoform and blood group					
			sm			fm		
			O	A	B	O	A	B
1260	2 HexNAc, 3 Hex, Fuc, HexNAcol			x	x			x
1301	Hex-HexNAc-**(HexNAc-(Fuc-)Hex-HexNAc-)**HexNAcol	A		x				
1349	Fuc-Hex-3**(Hex-(Fuc-)Hex-(Fuc-)HexNAc-6)**HexNAcol	B, H			x			
1365	HexNAc, 4 Hex, 2 Fuc, HexNAcol				x			
1390	2 HexNAc, 2 Hex, 3 Fuc, HexNAcol		x					
1390	Fuc-Hex-3**(HexNAc-(Fuc-)Hex-(Fuc-)HexNAc-6)** HexNAcol	A, H		x				
1406	2 HexNAc, 3 Hex, 2 Fuc, HexNAcol		x	x	x			
1447	HexNAc-(Fuc-)Hex-HexNAc-3 **(Fuc-Hex-HexNAc-6)**HexNAcol	A, H		x				
1552	2 HexNAc, 3 Hex, 3 Fuc, HexNAcol			x				
1593	HexNAc-(Fuc-)Hex-3**(HexNAc-(Fuc-) Hex-(Fuc-)HexNAc-6)**HexNAcol	A		x				
1698	2 HexNAc, 3 Hex, 4 Fuc, HexNAcol		x					
$[M–2H]^{2–}$								
938	2 HexNAc, 5 Hex, 3 Fuc, HexNAcol				x			
979	4 HexNAc, 3 Hex, 3 Fuc, HexNAcol			x				
1039	3 HexNAc, 5 Hex, 3 Fuc, HexNAcol				x			
1052	4 HexNAc, 3 Hex, 4 Fuc, HexNAcol			x				
1081	5 HexNAc, 3 Hex, 3 Fuc, HexNAcol			x				
1161	5 HexNAc, 4 Hex, 3 Fuc, HexNAcol			x				
1234	5 HexNAc, 4 Hex, 4 Fuc, HexNAcol			x				
1336	6 HexNAc, 4 Hex, 4 Fuc, HexNAcol			x				
	Acidic sequences							
$[M–H]^{–}$								
667	HexNAc, Hex, HSO_3, HexNAcol					x		x
675	Hex-(NeuAc-)HexNAcol			x	x	x		x
675	NeuAc-Hex-3HexNAcol					x		x
813	HSO_3-Hex-(Fuc-)HexNAc-HexNAcol	Su-Le						x
829	Hex-3**(HSO_3-Hex-HexNAc-6)**HexNAcol					x	x	x
878	HexNAc, Hex, NeuAc, HexNAcol		x	x	x	x		
958	HexNAc, Hex, NeuAc, HSO_3, HexNAcol					x		

Table I. continued

Molecular ion (observed)	Neutral sequences	Blood group sequences	Glycoform and blood group					
			sm			fm		
			O	A	B	O	A	B
966	NeuAc-Hex-3(NeuAc-)HexNAcol				x			
975	HexNAc, 2 HexFuc, HSO_3, HexNAcol	H				x	x	x
975	Hex-(Fuc-)Hex-(HSO_3-)HexNAc-HexNAcol	B						x
1032	2 HexNAc, 2 Hex, HSO_3, HexNAcol					x		x
1040	HexNAc, 2 Hex, NeuAc, HexNAcol		x	x	x			
1120	HexNAc, 2 Hex, NeuAc, HSO_3, HexNAcol			x	x	x		x
1121	HexNAc, 2 Hex, 2 Fuc, HSO_3, HexNAcol		x			x	x	
1121	Fuc-Hex-3**(Hex-(Fuc-)(HSO_3-)HexNAc-6)**HexNAcol	H, Su-Le						x
1137	HexNAc, 3 Hex, Fuc, HSO_3, HexNAcol							x
1178	2 HexNAc, 2 Hex, Fuc, HSO_3, HexNAcol					x	x	x
1186	NeuAc-Hex-**(Fuc-Hex-4HexNAc-6)**HexNAcol	H	x	x				
1186	HexNAc, 2 Hex, Fuc, NeuAc, HexNAcol			x	x			
1266	HexNAc, 2 Hex, Fuc, NeuAc, HSO_3, HexNAcol		x			x	x	x
1267	Fuc-Hex-3**(Fuc-Hex-(Fuc-)(HSO_3-)HexNAc-6)** HexNAcol	H, Su-Le				x		
1283	HexNAc, 3 Hex, 2 Fuc, HSO_3, HexNAcol							x
1324	2 HexNAc, 2 Hex, 2 Fuc, HSO_3, HexNAcol		x			x	x	x
1332	HexNAc, 2 Hex, 2 Fuc, NeuAc, HexNAcol			x	x			
1332	NeuAc-Hex-(Fuc-Hex-(Fuc-)HexNAc-)HexNAcol	H, Le	x					
1340	2 HexNAc, 3 Hex, Fuc, HSO_3, HexNAcol				x			
1340	HSO_3-Hex-HexNAc-(Hex-(Fuc-)Hex-HexNAc) HexNAcol	B						x
1381	HSO_3-Hex-HexNAc-(HexNAc-(Fuc-) Hex-HexNAc-)HexNAcol	A		x			x	
1477	NeuAc-Hex-3**(NeuAc-Hex-(Fuc-)HexNAc-6)**HexNAcol	Si-Le		x			x	
1486	2 HexNAc, 3 Hex, 2 Fuc, HSO_3, HexNAcol				x		x	
1527	Fuc + HSO_3-Hex-HexNAc-(HexNAc-(Fuc-) Hex-HexNAc-)HexNAcol			x			x	
1535	2 HexNAc, 2 Hex, 2 Fuc, NeuAc, HexNAcol		x					
1535	NeuAc-Hex-3**(HexNAc-(Fuc-)Hex-(Fuc-) HexNAc-6)**HexNAcol	A, Le		x				
$[M–2H]^{2–}$								
629	2 HexNAc, 2 Hex, Fuc, 2 HSO_3, HexNAcol					x		
702	2 HexNAc, 2 Hex, 2 Fuc, 2 HSO_3, HexNAcol					x		

Table I. continued

Molecular ion (observed)	Neutral sequences	Blood group sequences	Glycoform and blood group					
			sm			fm		
			O	A	B	O	A	B
710	2 HexNAc, 3 Hex, Fuc, 2 HSO_3, HexNAcol					x	x	x
783	2 HexNAc, 3 Hex, 2 Fuc, 2 HSO_3, HexNAcol					x	x	x
811	3 HexNAc, 3 Hex, Fuc, 2 HSO_3, HexNAcol					x		x
856	2 HexNAc, 3 Hex, 3 Fuc, 2 HSO_3, HexNAcol					x		
877	3 HexNAc, 3 Hex, Fuc, NeuAc, HexNAcol			x				
884	3 HexNAc, 3 Hex, 2 Fuc, 2 HSO_3, HexNAcol					x	x	
892	3 HexNAc, 4 Hex, Fuc, 2 HSO_3, HexNAcol						x	
958	3 HexNAc, 3 Hex, 3 Fuc, 2 HSO_3, HexNAcol					x		
965	3 HexNAc, 4 Hex, 2 Fuc, 2 HSO_3, HexNAcol					x	x	x
973	3 HexNAc, 5 Hex, Fuc, 2 HSO_3, HexNAcol							x
1039	3 HexNAc, 4 Hex, 3 Fuc, 2 HSO_3, HexNAcol						x	x
1112	3 HexNAc, 4 Hex, 4 Fuc, 2 HSO_3, HexNAcol							x

The assumptions are Hex-HexNAcol = core 1 (Hex-3HexNAcol); Hex-(HexNAc-)HexNAcol = core 2 (Hex-3(HexNAc-6)HexNAcol, C-6 branches in bold; all deoxyhexoses are fucose.
Fuc, fucose; Hex, Hexose, HexNAc, N-acetylhexosamine; HexNAcol, N-acetylhexosaminitol; NeuAc, N-acetylneuraminic acid; Le, Lewis; Su-Le, Sulfo Lewis; Si-Le, Sialyl Lewis; sm, slow-migration MUC5B; fm, fast-migrating MUC5B.

Table II. Blood group epitopes and the corresponding sequences detected by mass spectrometry.

	Blood group epitope	Observed sequence[a]
H	Fuc(α1-2)Galβ-	dHex-Hex-
A	GalNAc(α1-3)Galβ-	HexNAc-Hex-
	/	/
	Fuc(α1-2)	dHex
B	Gal(α1-3)Galβ-	Hex-Hex-
	/	/
	Fuc(α1-2)	dHex
Lewis	Gal(β1-3/4)GlcNAcβ-	Hex-HexNAc-
	/	/
	Fuc(α1-3/4)	dHex

[a]The fragmentation pattern of oligosaccharides with H, A, and B epitopes is published elsewhere (Schulz *et al.*, 2002a; Karlsson *et al.*, 2004). dHex, deoxyhexose; Hex, Hexose; HexNAc, *N*-acetylhexosamine.

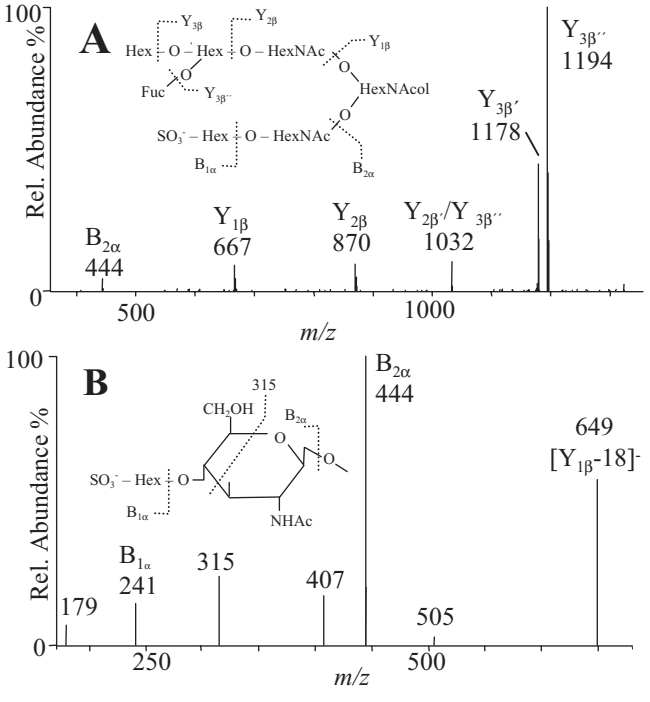

Fig. 3. Characterization of a major sulfated oligosaccharide with a blood group B sequence eluting as single peak at *m/z* 1340, purified from a fast-migrating (fm) MUC5B glycoform of a blood group B individual. Tandem mass spectrometry (MS/MS) spectrum is shown in (**A**), together with MS^3 of the daughter ion at *m/z* 667 (**B**).

Figure 4A shows the MS/MS spectrum of the blood group A-specific tetrasaccharide. Diagnostic fragments are found at *m/z* 512 ($Z_{2\alpha}$), which is indicative of loss of a HexNAc in a terminal position, *m/z* 625 ($[M–H]^-$ –108), and *m/z* 510 (B_2), where the latter two are indicative of a singly branched HexNAcol. The fragment at *m/z* 625 originates from a cleavage within the HexNAcol moiety (Karlsson *et al.*, 2004). Figure 4B shows the MS/MS spectrum of the blood group B-specific pentasaccharide, (Hex-(Fuc-)Hex-4HexNAc-HexNAcol), where *m/z* 715 origins from loss of a Hex in terminal position, and the two fragments at *m/z* 787 ($[M–108]^-$) and *m/z* 672 (B_3) are diagnostic for a singly branched HexNAcol. The fragment at *m/z* 571 results from a cleavage within the HexNAc moiety followed by loss of H_2O and is diagnostic for a type 2 branch.

The presence of ABO blood group-specific sequences on salivary MUC5B matching the blood group status suggests that 23 individuals were secretors. The six remaining individuals did not express the blood group-related sequences of *m/z* 733 or *m/z* 895, nor did we find any additional blood group sequences on other oligosaccharides in these samples. This finding is consistent with a nonsecretor status of these six individuals.

Increased sialylation on MUC5B from individuals mass spectrometrically identified as nonsecretors

The alcian blue-stained MUC5B component from a blood group B individual identified as a nonsecretor by MS is shown in Figure 5. Oligosaccharides were released from the positions marked in the inserted picture, and the total ion mass spectra from the upper (sm) and lower (fm) band are shown in Figure 5A and B, respectively. The sm components were dominated by sialylated oligosaccharides, whereas the fm glycoform contained mainly sulfated oligosaccharides, of which several abundant peaks contained two sulfate groups. The composition of detected molecular ions and the elucidated oligosaccharide sequences corresponding to the sm component in Figure 5A are listed in Table IV. All major peaks contained sialic acid and Fuc. Five neutral and two sialylated sequences were deduced. The highly abundant sialylated ion at *m/z* 1477 eluted as a single peak in the chromatogram indicating that it was only present as one component. It was identified as a doubly sialylated heptasaccharide with the sequence NeuAc-Hex-(NeuAc-Hex-(Fuc-)HexNAc)HexNAcol. The sequence indicates that Sialyl Lewis x (Si-Le^x) [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-] and/or Sialyl Lewis a (Si-Le^a) [NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-] epitopes are expressed on MUC5B. Four secretors and four nonsecretors were screened for binding of antibodies against these two epitopes. Western blots showed that binding to Si-Le^x and Si-Le^a was found both among secretors and nonsecretors where MUC5B positive bands were found, and that the binding to Si-Le^a was much stronger in nonsecretors compared with secretors (Figure 6). We could therefore conclude that the increased sialylation among nonsecretor individuals was linked to an increased expression of Si-Le^a epitopes. The western blots also indicate that MUC7 and other lower molecular weight glycoproteins preferentially expressed Si-Le^x and were less affected by the secretor status.

To verify the observation that individuals identified as nonsecretors carried more sialylated oligosaccharides compared with secretors, we reanalyzed oligosaccharides from 13 secretors and four nonsecretors, this time obtained from PVDF strips containing the complete MUC5B bands. The relative amounts of Fuc, Hex, HexNAc, NeuAc, and sulfate in each sample were calculated based upon the identification

of the composition of molecular ions and their relative peak intensity. The results are shown in Figure 7 and support our observation that the amounts of sialic acid residues are increased relative to other saccharide residues on nonsecretor MUC5B.

Discussion

The presence of MUC5B glycoforms in saliva has previously been established with biochemical methods and antibodies (Bolscher *et al*., 1995; Wickström *et al*., 1998; Thornton *et al*., 1999). The two salivary MUC5B bands found in this study from three individuals of blood group A, B, and O contained predominantly either neutral or sulfated oligosaccharides as judged from relative intensities in the mass spectra and always had sialylated components present. The total ion spectra were different between the individuals, and our data suggest that one explanation for this is that the different blood group-specific sequences were frequently expressed. The observation of ABO blood group-specific epitopes on salivary MUC5B is consistent with other studies using immunochemical techniques (Prakobphol *et al*., 1993). Mixtures of neutral and sialylated MUC5B oligosaccharides from blood group O secretor individuals have been previously characterized (Klein *et al*., 1992; Thomsson *et al*., 2002) and were described as large, extensively fucosylated and with frequently expressed H epitopes (Fucα1-2Galβ1-), in agreement with our results. The presence of sulfated glycoforms and epitopes has also been determined with immunochemical methods, by using an antibody that recognizes the HSO_3-3Galβ1-3GlcNAcβ1- moiety of sulfo Lewis a (Su-Le^a) epitopes (Veerman *et al*., 1997).

We used a mass spectrometric approach to screen for the presence of blood group sequences in MUC5B. Six of

Table III. ABH salivary sequences found in isoforms of tetrasaccharides at *m/z* 733 and pentasaccharides at *m/z* 895 from 29 individuals.

		Number of positive salivary phenotypes				
ABO blood group	n	—	H	HA	HB	HAB
O	13	4	9			
A	7	1		6		
B	6	1			5	
AB	3					3
Total	29	6			23	

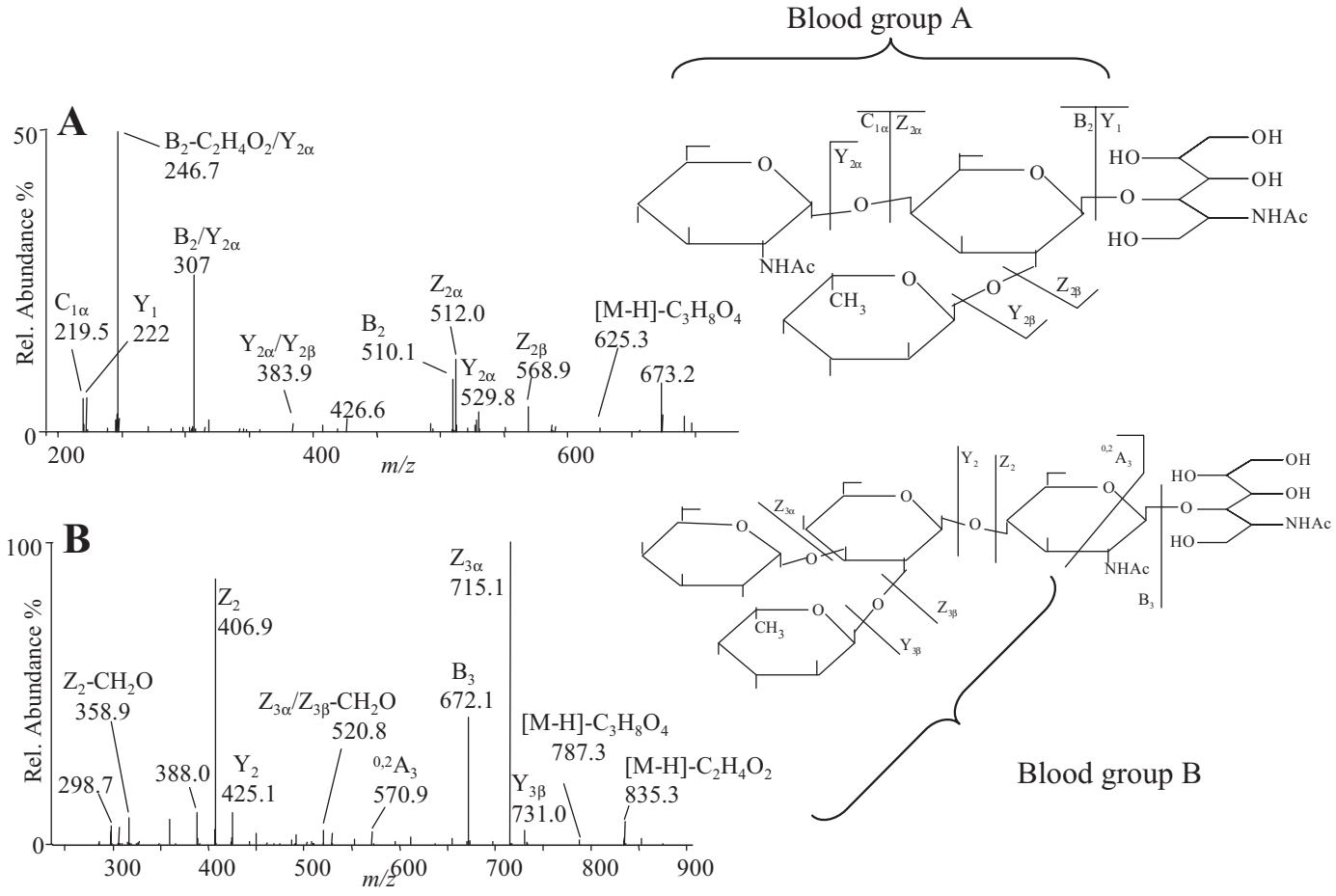

Fig. 4. Tandem mass spectrometry (MS/MS) spectrum of a blood group A tetrasaccharide with $[M–H]^-$ of *m/z* 733 and the sequence HexNAc-(Fuc-)Hex-HexNAcol (**A**) and a blood group B pentasaccharide with $[M–H]^-$ of *m/z* 895 and the sequence Hex-(Fuc-)Hex-HexNAc-HexNAcol (**B**).

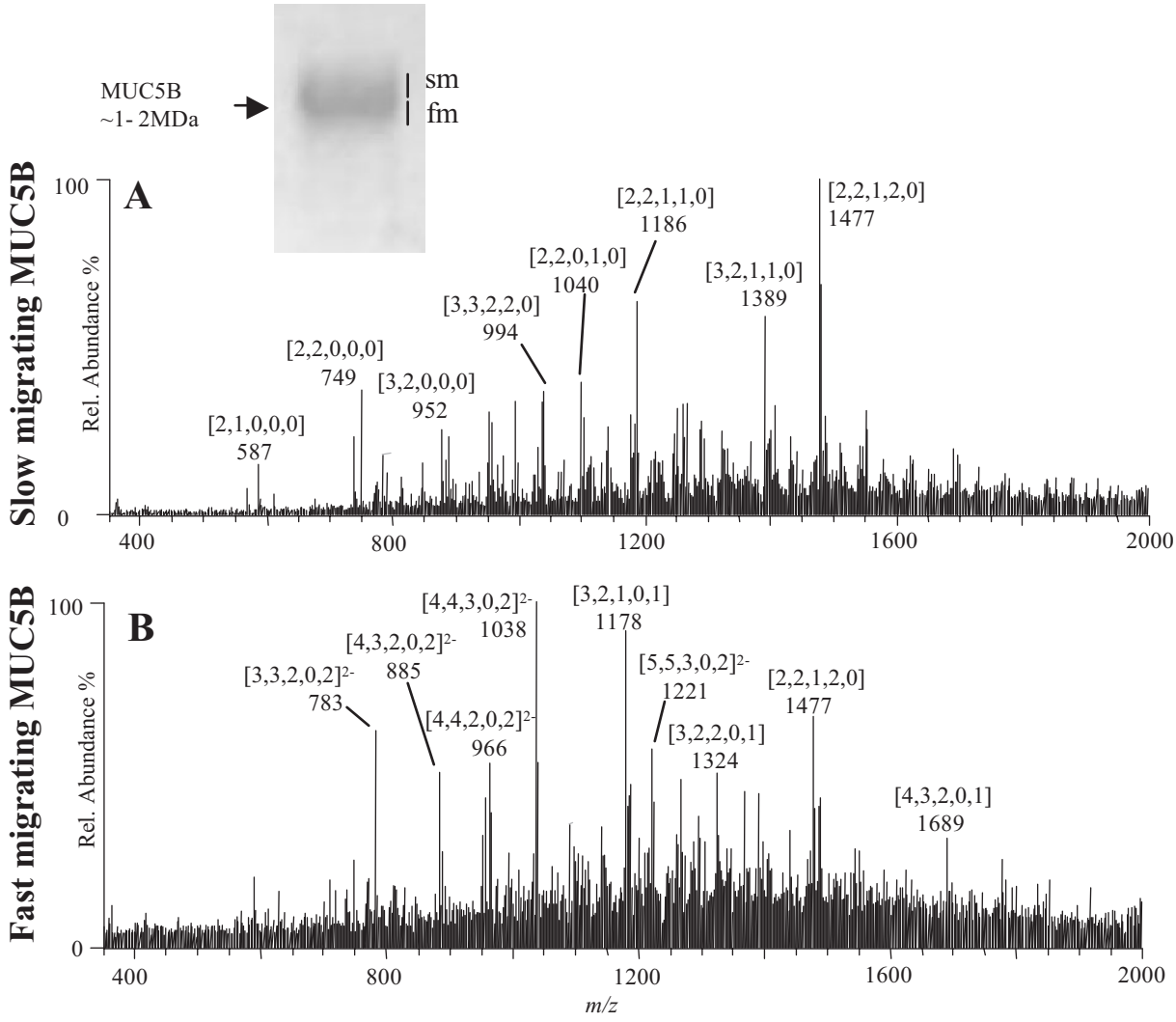

Fig. 5. MUC5B from saliva of a nonsecretor blood group B individual, separated by sodium dodecyl sulfate-agarose/polyacrylamide composite gel electrophoresis (SDS–AgPAGE), blotted to polyvinylidene fluoride (PVDF) membrane, and stained with alcian blue. Total ion mass spectra from liquid chromatography–mass spectrometry (LC-MS) of O-linked oligosaccharides released from a slow migrating (sm) (**A**) and fast migrating (fm) (**B**) glycoform originating as indicated in the inserted picture of the MUC5B band. Peaks are labeled with *m/z*, charge state if *z* is >1 and monosaccharide composition [*N*-acetylhexosamine (HexNAc), Hexose (Hex), fucose (Fuc), *N*-acetylneuraminic acid (NeuAc), and sulfate]. For simplicity, the terminal *N*-acetylhexosaminitol (HexNAcol) is included as an HexNAc. Compositions and elucidated sequences of oligosaccharides in the sm component are listed in Table IV.

29 individuals did not express any ABH blood group-related epitopes. They were all of Caucasian origin and made up 27% of the Caucasians that took part in the study. This figure is close to the reported 20% of nonsecretors in the Caucasian population not encoding for the Se α2fucosyltransferase (FUT2) that makes the majority of the H epitopes (and consequently A and B) in saliva (reviewed by Henry *et al.*, 1995), and suggest that MS can be used as a supplementary technique for determining the secretor status of an individual.

Mass spectra of MUC5B oligosaccharides obtained from the nonsecretors suggested that these individuals expressed more sialylated oligosaccharides than secretors. We found that increased sialylation of oligosaccharides obtained from nonsecretors was linked to increased expression of the saccharide epitope Si-Le^a. This finding is supported by a previous study, where whole saliva was analyzed from individuals genotyped for secretor status, by using a monoclonal antibody (mA) against Si-Le^a (Brockhaus *et al.*, 1985). Their hypothesis for increased expression of Si-Le^a epitopes was that when the Se gene product (FUT2) is lacking, Galβ1-3GlcNAc precursor chains that otherwise would have been converted into H type 1 chains [Fucα1-2Galβ1-3GlcNAc] are instead available for conversion into Si-Le^a [NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAc]. This hypothesis is supported by this study. Further support that a sialyltransferase substitute for the fucosyltransferase in nonsecretors comes from the analysis of two Asian participants in this study, who were identified as B secretors, but whose MUC5B oligosaccharide mass profile resembled the characteristic nonsecretor profile of predominantly sialylated oligosaccharides. Semiquantitative analyses performed on sm and

Table IV. Oligosaccharides from molecular ions from a salivary MUC5B glycoform of a blood group B nonsecretor individual (Figure 4A), analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Molecular ion (observed)	Sequences	Blood group sequences
$[M–H]^-$		
587	Hex-HexNAc-HexNAcol	
749	HexNAc, 2 Hex, HexNAcol	
749	Hex-**(Hex-4HexNAc-6)**HexNAcol	
878	HexNAc, Hex, NeuAc, HexNAcol	
952	Hex-HexNAc-3**(Hex-HexNAc-6)**HexNAcol	
975	Hex-(Hex-(HSO_3-)(Fuc-)HexNAc-)HexNAcol	Le
975	Hex-(HSO_3-)(Fuc-)HexNAc-Hex-HexNAcol	Le
1040	HexNAc, 2 Hex, NeuAc, HexNAcol	
1098	Hex-(Fuc-)HexNAc-3**(Hex-HexNAc-6)**HexNAcol	Le
1178	2 HexNAc, 2 Hex, Fuc, HSO_3, HexNAcol	
1186	HexNAc, 2 Hex, Fuc, NeuAc, HexNAcol	
1244	Hex-(Fuc-)HexNAc-3**(Hex-(Fuc-)HexNAc-6)**HexNAcol	Le
1244	2 HexNAc, 2 Hex, 2 Fuc, HexNAcol	
1260	Hex-(Fuc-)HexNAc-Hex-3**(Hex-HexNAc-6)**HexNAcol	
1266	HexNAc, 2 Hex, Fuc, NeuAc, HSO_3, HexNAcol	
1331	NeuAc-Hex-3**(NeuAc-Hex-HexNAc-6)**HexNAcol	
1389	2 HexNAc, 2 Hex, Fuc, NeuAc, HexNAcol	
1477	NeuAc-Hex-3**(NeuAc-Hex-(Fuc-)HexNAc-6)**HexNAcol	Si-Le
1486	2 HexNAc, 3 Hex, 2 Fuc, HSO_3, HexNAcol	
1551	2 HexNAc, 3 Hex, Fuc, NeuAc, HexNAcol	
$[M–2H]^{2-}$		
783	2 HexNAc, 3 Hex, 2 Fuc, 2 HSO_3, HexNAcol	
848	2 HexNAc, 3 Hex, 2 Fuc, NeuAc, HexNAcol	
888	2 HexNAc, 3 Hex, 2 Fuc, NeuAc, HSO_3, HexNAcol	
994	2 HexNAc, 3 Hex, 2 Fuc, 2 NeuAc, HexNAcol	
958	3 HexNAc, 4 Hex, Fuc, NeuAc, HexNAcol	
1031	3 HexNAc, 4 Hex, 2 Fuc, NeuAc, HexNAcol	
1039	3 HexNAc, 4 Hex, 3 Fuc, 2 HSO_3, HexNAcol	
1104	3 HexNAc, 4 Hex, Fuc, 2 NeuAc, HexNAcol	
1177	2 HexNAc, 3 Hex, 2 Fuc, 3 NeuAc, HexNAcol	
1249	3 HexNAc, 4 Hex, 3 Fuc, 2 NeuAc, HexNAcol	
1322	3 HexNAc, 4 Hex, 2 Fuc, 3 NeuAc, HexNAcol	

The secretor status was based upon the observation that no blood group-related sequences were identified by mass spectrometry in this saliva sample. The assumptions are Hex-HexNAcol = core 1 (Hex-3HexNAcol); Hex-(HexNAc-)HexNAcol = core 2 (Hex-3**(HexNAc-6)**HexNAcol), C-6 branches in bold; all deoxyhexoses are fucose.
Fuc, fucose; Hex, Hexose, HexNAc, *N*-acetylhexosamine; HexNAcol, *N*-acetylhexosaminitol; NeuAc, *N*-acetylneuraminic acid; Le, Lewis; Si-Le, Sialyl Lewis.

fm bands from these individuals gave values of above 0.5 NeuAc per average oligosaccharide, which were typical of nonsecretors (Figure 7). The "partial" secretor phenotype Le(a+b+) involving a weak secretor gene is rare in Caucasians, but common in other populations such as Asians (Broadberry and Lin-Chu, 1991).

Our results highlight that there is extensive natural variation in the glycosylation of MUC5B oligosaccharides, which is partly dependent on inherited factors such as blood group and secretor status. There are further known inherited factors that could be expected to influence the individual variation of MUC5B glycosylation, which we have not investigated in this work. One factor is ABO polymorphism, with six known, common phenotypes in Caucasians (A_1, A_2, B, A_1B, A_2B, and O), giving rise to ABO blood group-specific glycosyltransferases of varying efficiency. Also, the Lewis histo-blood group antigens are found in secretions, and the phenotype can be confirmed in

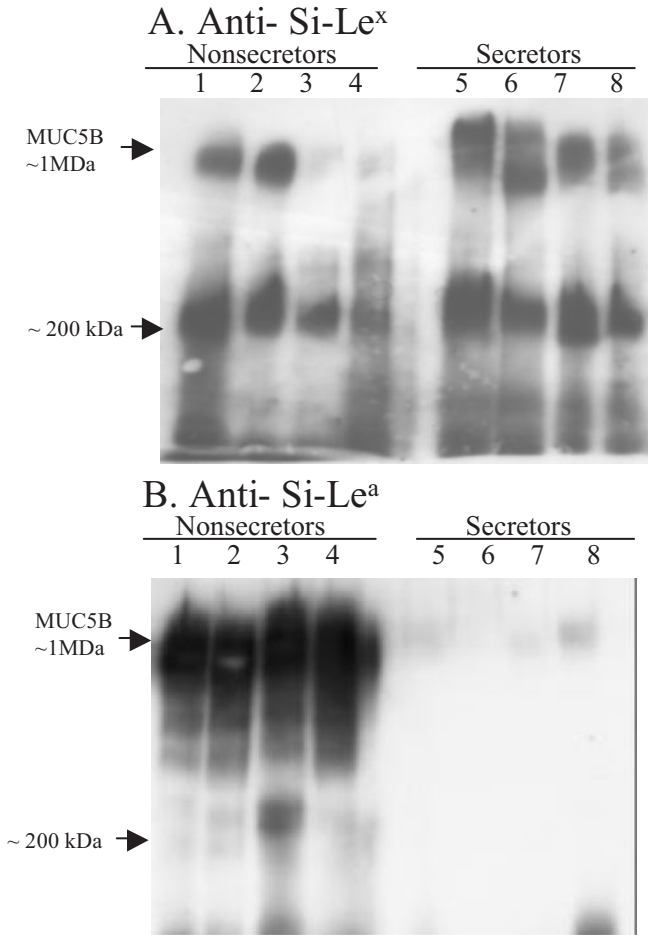

Fig. 6. MUC5B from eight individuals previously determined as secretors and nonsecretors with mass spectrometry (MS) was tested for binding with antibodies against the oligosaccharide epitopes Sialyl Lewis x (Si-Le^x) (**A**) and Sialyl Lewis a (Si-Le^a) (**B**).

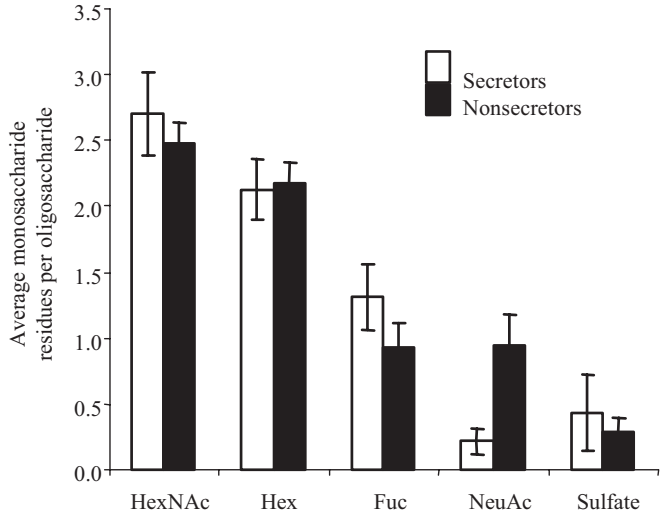

Fig. 7. Relative amounts of monosaccharide residues on MUC5B derived oligosaccharides from secretors and nonsecretors (secretor status determined by mass spectrometry, see text). The calculation was based upon relative intensities of oligosaccharide peaks in liquid chromatography–mass spectrometry (LC-MS) chromatograms (see Materials and methods). Student *t*-test showed a significant difference in sialic acid levels ($p = 1.7 \times 10^{-7}$).

saliva with antibodies against the Lewis antigens Le^a [Galβ1-3(Fucα1-4)GlcNAcβ1-] and Le^b [Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-]. The Lewis fucosyltransferase (FUT3) is expressed in ~93% of the Caucasian population. In the approach presented in this study, we cannot mass spectrometrically distinguish between the structural isomers Le^a/Le^x and Le^b/Le^y.

We have noticed that salivary MUC5B oligosaccharide profiles can be heterogenous even among individuals of the same blood group. The statistical data presented in this work suggest that the relative amount of sulfation on the MUC5B glycoform can vary extensively between individuals and may account for some of the heterogeneity between individuals with the same blood group. Different MUC5B glycoforms are produced by salivary glands that are physically separated in the oral cavity (Veerman *et al.*, 1992; Bolscher *et al.*, 1995; Wickström *et al.*, 1998; Thornton *et al.*, 1999), so it is possible that there could be subsets of cells specialized to produce and secrete either neutral or sulfated MUC5B glycoforms.

The clinical impact of carrying blood group-dependent glycosylation on mucosal surfaces has been investigated by many researchers. ABO blood group, secretor phenotype, and/or Lewis blood group-status have been linked with predisposition to bacterial infections by, for example *Helicobacter pylori* and *Candida sp.*, and with chronic urinary tract infection (reviewed by D'Adamo and Kelly, 2001). Further evidence for a protective role for the Se gene is in FUT2 knockout mice, which show increased susceptibility to cervical yeast infection (Hurd and Domino, 2004). The analytical approach applied in this work permits potential screening of groups of individuals to investigate if a predisposition to a specific disease can be linked not only to known phenotypes such as ABO blood group and secretor phenotype, but also to combinations of phenotypes resulting from the activity of other glycosyltransferases such as fucosyl-, sialyl- or sulfotransferases.

The variation in salivary MUC5B glycosylation may have profound effects on the microenvironment in the oral cavity of different individuals. We propose that the glycosylation variation of MUC5B between individuals is due to territorial and historical diversity of the biological pressure from pathogens. This would suggest that salivary MUC5B is a key molecule in the initial interaction between host and invading pathogen and is important for understanding the mechanism of mucosal defense. In addition, our results also provide a warning that when comparative studies are performed on mucin glycosylation or bacterial infection in healthy and diseased states, the possibility that individuals can vary considerably even among the healthy controls should be considered carefully.

Materials and methods

Sample collection and preparation

Twenty-nine human volunteers (22 Caucasian, 7 Asian) with known ABO blood group status were encouraged to

spit saliva (300–600 μL) produced by the glands under the tongue (submandibular/sublingual glands) into a test tube. Aliquots of 200 μL saliva were reduced within 60 min after collection with 200 μL of sample buffer containing dithiothreitol (20 mM), 20% glycerol, Tris–HCl (0.75 M, pH 8.1), 2% SDS, and 0.01% bromophenol blue, for 20 min at 100°C. The samples were alkylated with 25 mM of iodoacetamide overnight, in the dark and with gentle shaking. The samples were centrifuged at 10,000 × *g* for 10 min to precipitate nonreduced material (usually no pellet was observed) and then concentrated to 15–40 μL on 100 kD cut-off membranes (Millipore, Bedford, MA). Dilution of each sample was performed by adding 120 μL of sample loading buffer (as above, but with 30% glycerol), and 40 μL loaded onto 0–7% acrylamide/1% agarose gels (Schulz *et al*., 2002a).

The gels were run in boronate/Tris buffer (192 mM boric acid, pH adjusted to 7.6 with Tris, 1 mM EDTA, and 0.1% SDS) at 30 mA/gel for 3–3.5 h until the dye front ran out of the gel. The gels were blotted to PVDF membranes and stained with alcian blue as described by Schulz *et al.* (2002a). The presence and identification of MUC5B as the dominating mucin in the 1–2 MDa fraction was done by using western blotting (Thomsson *et al*., 2002) and peptide mass fingerprinting (Schulz *et al*., 2004).

Release of O-linked oligosaccharides

Reductive β-elimination of oligosaccharides from mucins on blots was done essentially as described by Schulz *et al.* (2002a), with minor alterations. Briefly, alcian blue stained PVDF membrane bands were cut out either as strips across the whole MUC5B band (~2 mm wide and 15 mm long) for semiquantitative analyses or as square bands (~10–20 mm^2) for glycoform analyses. The oligosaccharides were released overnight in 50 mM NaOH and 0.5M $NaBH_4$ and desalted on 25 μL cation exchange resin in a C18 ZipTip (Millipore), followed by vacuum evaporation and removal of borate with repetitive acidic methanol extraction.

LC/MS, MS/MS, and MS^3 of released oligosaccharides

Sample injection and LC was performed by using a Surveyor autosampler and LC pump (Thermo Finnigan, San Jose, CA). Oligosaccharides were resuspended in 10 μL of water and injected on graphitized carbon columns, either commercially packed columns (100 × 0.32 mm or 100 × 0.18 mm id, 7 μm Hypercarb particles, Thermo-Hypersil, Runcorn, UK) or homepacked with the same particles. Oligosaccharides were eluted with an H_20/acetonitrile gradient containing 10 mM NH_4HCO_3 (0–34% acetonitrile in 27 min, followed by 34–90% acetonitrile in 6 min, then a 7-min wash with 90% acetonitrile). MS was performed by using an LCQ Deca XP mass spectrometer (Thermo Finnigan) in negative mode. The capillary temperature was 340°C, the capillary voltage 59 V, and the electrospray voltage 3.5 V. For MS/MS and MS^3 experiments, the normalized collision energy was 40% with an activation time of 30 min. For MS/MS experiments, MS was performed with four scan events: full scan with mass range *m*/*z* 350–2000, followed by successive MS/MS scans after collision induced fragmentation for the three most intense ions in each full scan. For MS^3 experiments, a three-scan method was used, full scan *m*/*z* 350–2000, the data-dependent MS/MS scan on the most intense peak, followed by data-dependent MS^3 of the most intense fragment in the mass range *m*/*z* 400–840.

Semiquantitative analyses using MS

For semiquantitation experiments, alcian blue-stained PVDF membrane strips were cut out across the whole MUC5B band, ~2 mm wide and 15 mm long, and the oligosaccharides released as described above. The LC-MS conditions were as described above, except for that a single scan method was applied, with a full scan in the mass range 350–2000 *m*/*z*. Thirteen secretors and four nonsecretors were analyzed, with the following blood group distribution: O : A : B : AB, 3:5:3:2 (secretors) and 2:1:1:0 (nonsecretors). The chromatograms were processed by using the program ACD/Spec Manager (ACD/Laboratories, Toronto, Ontario) for peak picking. Identification of oligosaccharide ions and calculation of relative amounts of monosaccharide residues were performed in an application program developed at Proteome Systems Limited (Sydney, Australia). The matches were manually examined, and in cases when different molecular ion compositions were possible, the correct composition was chosen based upon MS/MS data from previous analyses.

Western blot with antibodies raised against carbohydrate epitopes Si-Le^a and Si-Le^x

Reduced and alkylated saliva (equivalent of 0.06 μL saliva in 5–10 μL sample buffer for the Si-Le^a assay and equivalent of 6 μL saliva in 5–10 μL sample buffer for the Si-Le^x assay) was analyzed on gels and blotted to PVDF membranes as described above. The blots were wetted in methanol and blocked overnight at 4°C in 1% bovine serum albumin (BSA) and in phosphate buffered saline-T (PBS-T; PBS + 0.1% Tween-20), followed by incubation for 1–2 h at room temperature with antibodies against either Si-Le^a (C241, Nilsson *et al*., 1987) or Si-Le^x (CSLEX-1 from A.T.C.C., Manassas, VA), both diluted 1 μg/mL in blocking solution. After three washes with PBS-T, the membranes were incubated with horse radish peroxidase-conjugated goat anti-mouse IgG (Chemicon, Victoria, Australia) diluted 1:10,000 in blocking solution for 60 min at room temperature. The membranes were washed five times for 5 min in PBS-T, followed by incubation (2 min) in Supersignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL). The blots were exposed to Hyperfilm ECL (Amersham Bioscience, Little Chalfont, UK) for 1–30 s and developed by using Phenisol developer and Hypam rapid fixer solution from Ilford Imaging (Cheshire, UK).

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Abbreviations

Fuc, fucose; Hex, Hexose; HexNAc, *N*-acetylhexosamine; HexNAcol, *N*-acetylhexosaminitol; LC, liquid chromatography; MS, mass spectrometry; NeuAc, *N*-acetylneuraminic acid; PBS, phosphate buffered saline; SDS–AgPAGE, sodium dodecyl sulfate-agarose/polyacrylamide composite gel electrophoresis; Si-Le^a, Sialyl Lewis a; Si-Le^x, Sialyl Lewis x.

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