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Rat Gastric Mucins Recognized by Monoclonal Antibodies RGM21 and HIK1083: Isolation of Mucin Species Characteristic of the Surface and Glandular Mucosa[1]

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Whole mucins and reduced subunits were extracted from the corpus of the rat stomach. After purification by Sepharose CL-4B chromatography followed by cesium trifluoroacetate equilibrium centrifugation, they were analyzed by Sepharose CL-2B chromatography, rate-zonal sedimentation centrifugation, and Q-Sepharose chromatography. Monoclonal antibodies RGM21 and HIK1083, which histochemically stained mucins in the surface and glandular mucosa of the rat stomach, respectively, were used to detect the site-specific mucins. Although RGM21- and HIK1083-reactive mucins both had a multimerized structure, the density and size of both the whole mucins and reduced subunits differed, thus indicating the presence of distinct mucin species in the surface and glandular mucosa. The mucin subunits were separated into four fractions, UB, B1, B2a, and B2b, by Q-Sepharose chromatography. HIK1083 reacted mainly with UB, while RGM21 reacted with B1, B2a, and B2b. These results, combined with dot-blot, amino acid, and carbohydrate composition analyses, showed that the surface mucins may consist of three kinds of subunits. In contrast, the glandular mucins may consist of one kind of subunit which differs from that of surface mucins.

Key words: gastric mucin, glandular mucosa, monoclonal antibody, surface mucosa.

The surface of the stomach is coated with mucus, which protects the mucosa from gastric acid, pepsin, and pathogens. Mucins, the major component of mucus, are believed to contribute to this function. They are synthesized and secreted by two types of cells, surface mucus cells and glandular mucus cells (mucus neck cells), in the corpus of the stomach (*1, 2*). These mucin species have been distinguished histochemically: for example, surface mucins are stained by galactose oxidase-cold thionine Schiff (GOCTS) staining, while glandular mucins are stained by paradoxical concanavalin A staining (PCS) (*3, 4*). Thus, at least two mucin species bearing different glycoforms are believed to be present in the corpus of the stomach.

Many recent studies on mucin genes also indicated that different types of mucin gene are expressed in the surface and gland of the corpus mucosa of the human stomach. It was reported that the *MUC5AC* gene was expressed in the surface of the human stomach, while the *MUC6* gene was expressed in the glandular mucosa (*5-7*). Studies using gene technology have revealed important information. However, biochemical characterization of mucins is still needed, because mucin is also characterized by its carbohydrate portion. Indeed, there are cell-specific glycoforms, as mentioned already.

Although biochemical characterization of gastric mucins is difficult because of their huge size and high carbohydrate content, several studies have been reported (*8-11*). For the characterization of mucin molecules, specific tools to recognize each mucin species are important. Recently, we established monoclonal antibodies (MAbs), each of which recognizes a specific carbohydrate portion of rat gastric mucins (*12, 13*). Histochemical studies showed that the monoclonal antibodies recognized mucins in a site-specific manner. For example, MAb RGM21 stained mucins in the surface mucosa, while HIK1083 (RGM41 in Ref. *13*) stained those in the glandular mucosa (*13, 14*). The former reacts with Gal and/or GalNAc residues (*13*), and the latter with peripheral α-GlcNAc residues (*15*). Therefore, these MAbs were expected to be useful for detection of surface and glandular mucins individually.

In this paper, we report that RGM21-reactive surface mucins and HIK1083-reactive glandular mucins have different molecular structures and that three kinds of subunits exist in RGM21-reactive surface mucins.

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Abbreviations: ABTS, 2,2′-azino-di-[3-ethyl-benzthiazoline-6-sulphonate]; CsTFA, cesium trifluoroacetate; DTT, dithiothreitol; ELLA, enzyme-linked lectin binding assay; GOCTS, galactose oxidase-cold thionine Schiff; GuHCl, guanidinium hydrochloride; HRP, horseradish peroxidase; PAS, periodate-Schiff; PCS, paradoxical concanavalin A staining; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; UEA-I, *Ulex europeus* agglutinin I.

MATERIALS AND METHODS

Materials—The PD-10 column, Q-Sepharose FF, and Sepharose CL-2B and CL-4B were purchased from Pharmacia Biotech (Tokyo). Guanidinium hydrochloride (GuHCl, biochemical grade) and cesium trifluoroacetate (CsTFA) were purchased from Nakalai Tesque (Kyoto). The Centriprep 30 centrifugal concentrator was a product of Millipore (Bedford, USA). Monoclonal antibodies, RGM21 and HIK1083, were prepared as previously described (*13*). Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies were purchased from DAKO Japan (Kyoto), and HRP-conjugated UEA-I was purchased from Seikagaku Kogyo (Tokyo). The polyvinylidene difluoride (PVDF) membrane was purchased from Bio-Rad (Richmond, USA). Microtiter plates (96-well) were products of Sumitomo Bakelite (Tokyo).

Isolation of Mucins—Male Wistar rats (150-170 g) were killed under CO_2 and their stomachs were rapidly excised. After removal of the gel layer by gentle washing using 2% *N*-acetylcysteine in Dulbecco's phosphate-buffered saline (*16*), the corpus region was cut off. Mucosa was scraped off with a plastic plate and immediately placed in ice-cold 6 M GuHCl, pH 7.4, containing 2% (v/v) Triton X-100, 50 mM Tris, 10 mM EDTA, 2 mM *N*-ethylmaleimide, 2 mM benzamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.15 mM pepstatin A. When mucosa was extracted under reduced conditions, 0.1 M dithiothreitol (DTT) was added instead of *N*-ethylmaleimide. The mucosa was homogenized in a glass homogenizer very gently by hand at 0°C. The homogenates were stirred for 15 h at 4°C and centrifuged at 8,000×*g* for 60 min. The supernatants were loaded on a column (2.6 ×58 cm) of Sepharose CL-4B, and the fractions eluted near the void volume were pooled as mucin fractions for further studies. The supernatants obtained from the reduced extraction were alkylated with iodoacetamide before the chromatography.

Column Chromatography—Sepharose CL-4B chromatography was carried out using 4 M GuHCl, pH 7.4, containing 0.5% Triton X-100, 50 mM Tris, 10 mM EDTA, 2 mM benzamidine hydrochloride and 1 mM PMSF as an eluent. Each fraction collected was assayed for hexose, protein, periodate-Schiff (PAS)-stained materials, and MAbs-reactive materials as below. Sepharose CL-2B chromatography was done with a column (1.1×45 cm) using the same eluent as Sepharose CL-4B.

Anion exchange chromatography was carried out on a Q-Sepharose column (1.5×5 cm) equilibrated with 8 M urea, containing 30 mM Tris-HCl, pH 7.4, and 0.5% Triton X-100. The samples in GuHCl solution were concentrated by Centriprep 30, then GuHCl was replaced with urea by PD-10 column chromatography. The samples were loaded on a column and, after being washed with starting buffer, were eluted with a linear gradient of 30 mM Tris-HCl, pH 7.4, to 50 mM Tris-HCl, pH 7.4, containing 28 mM NaCl, in 8 M urea containing 0.5% Triton X-100, then with a linear gradient of 28-200 mM NaCl in 8 M urea containing 50 mM Tris-HCl, pH 7.4, and 0.5% Triton X-100. Fractions were collected, and urea was removed by precipitating the mucins with 71% ethanol containing 1.0 g potassium acetate/liter at 4°C overnight. The precipitates were redissolved in 4 M GuHCl, pH 7.4, containing 0.01% Triton X-100 and 50 mM Tris, then assayed for MAbs- and UEA-I-reactive mucins, and PAS- and high-ion diamine (HID)-stained mucins.

Centrifugal Analyses—CsTFA equilibrium centrifugation was performed at 152,000×*g* for 120 h at 10°C with a starting density of 1.40 g/ml in CsTFA/0.4 M GuHCl as previously described (*9*).

Rate-zonal sedimentation on a glycerol density gradient was performed as described (*17*). Samples were overlaid on a 12-ml linear gradient of glycerol (10 to 35%, w/v) formed in the 4 M GuHCl, pH 7.4, containing 0.5% Triton X-100 and 50 mM Tris on a cushion of 1 ml of 50% glycerol in the same solvent, then the gradients were centrifuged at 68,000×*g* for 24 h at 19°C.

Dot-Blot Analysis—Dot-blotting was performed as described previously (*9*). The samples were immobilized on a PVDF membrane by water suction. After washing, the membrane was stained with PAS or HID. The stained membrane was scanned by a computer-controlled photoscanner, and the densities were determined using NIH-Image computer software.

ELISA and ELLA—Each well of a microtiter plate was coated with the samples in 0.05 M carbonate-bicarbonate buffer, pH 9.6, and kept at 4°C for 2 d. After washing with 20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.02% Tween 20 (TBS-T) three times, the wells were blocked with 2% skimmed milk in TBS for 1 h at room temperature, then washed again. A solution of MAb, RGM21, or HIK1083, was then added and incubated for 1 h. HRP-conjugated goat anti-mouse immunoglobulin was used as second antibody, and ABTS-H_2O_2 solution was used for color development. The absorbancy at 405 nm was measured, and the tentative mucin concentration was calculated from a standard curve obtained using purified mucin. A standard curve was also obtained using the HIK1083-reactive mucin fraction, UB, described below. Apparent RGM21- or HIK1083-reactivity was calculated on the basis of the data that approximately 12% (w/w) of the total mucin subunits reacted only with HIK1083.

ELLA was performed similarly to ELISA except that HRP-conjugated UEA-I was used instead of MAb and HRP-conjugated second antibodies.

Chemical Analysis—Hexose was measured using the phenol/H_2SO_4 method (*18*), and protein concentration was determined using the BCA method (*19*) with a Pierce protein assay kit. Amino acid analysis was performed using a Waters Pico-Tag apparatus according to the manufacturer's procedures. Hexose analysis was performed by HPLC after pre-labeling of sugar with dabsyl hydrazine (*20*). Hexosamine analysis was performed as previously described (*21*). Sialic acid was determined by the methods of Hara *et al.* (*22*). For sugar analyses, the mucin samples were hydrolyzed with 2 M TFA at 100°C for 4 h for hexose, 4 M HCl at 100°C for 6 h for hexosamine, and 25 mM H_2SO_4 at 80°C for 1 h for sialic acid.

RESULTS

Extraction and Purification of the Rat Corpus Mucins—Whole mucins and reduced subunits were extracted from the corpus of rat stomach with buffered 6 M GuHCl containing 2% Triton X-100 and proteinase inhibitors in the

absence (under non-reduced conditions for the disulfide bonds) and presence (under reduced conditions) of DTT, respectively. The extracts were then applied onto a Sepharose CL-4B column. Macromolecules extracted under non-reduced conditions were detected in the void volume of the column by the phenol/H_2SO_4 method (Fig. 1A). The excluded fractions were stained by PAS; therefore, these fractions contained glycoconjugates. Almost all small proteins were retained on the column and were therefore separated from the large glycoconjugates by this procedure. MAbs RGM21 and HIK1083 detected the excluded fractions but not the other fractions (Fig. 1B). In the extracts

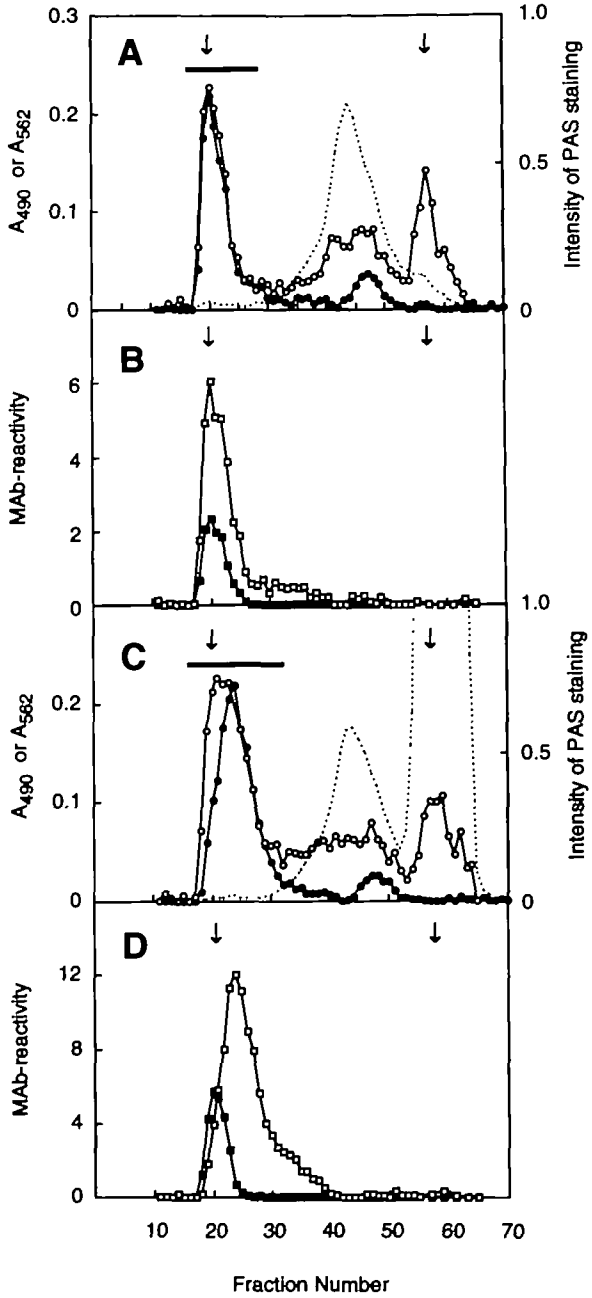

Fig. 1. **Sepharose CL-4B chromatography of the extracts from the corpus of rat stomach.** The corpus mucosa of rat stomach was extracted with 6 M GuHCl, pH 7.4, containing 2% Triton X-100, 50 mM Tris, and proteinase inhibitors with (C, D) or without (A, B) 0.1 M DTT as described in "MATERIALS AND METHODS". The extracts were loaded onto a column of Sepharose CL-4B, and each fraction was assayed for: (A, C), hexose (A_{490}, open circle), protein (A_{562}, dashed line), and PAS-stained materials (solid circle); (B, D), RGM21- (open square) and HIK1083- (solid square) reactive materials. The arrows indicate the void and total volume of the column. The fractions indicated by the bar were pooled and purified further.

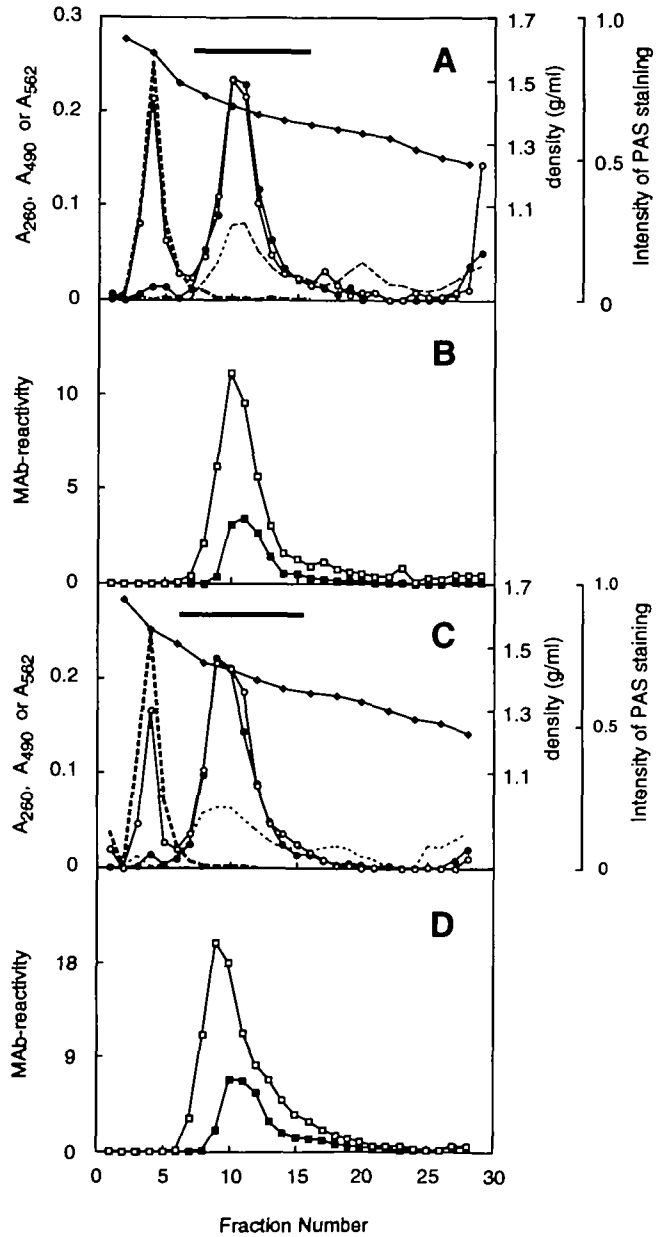

Fig. 2. **CsTFA equilibrium centrifugation of the macromolecules obtained from the extract in the absence (A, B) or presence (C, D) of DTT.** The macromolecules eluted near the void volume of a Sepharose CL-4B column were concentrated by use of a Centriprep 30 concentrator, dialyzed, then subjected to CsTFA equilibrium centrifugation. The initial density was adjusted to 1.40 g/ml in CsTFA/0.4 M GuHCl, and centrifugation was performed at 152,000 × *g* for 120 h at 10°C. Each fraction was assayed for: (A, C), density (diamond), hexose (A_{490}, open circle), protein (A_{562}, fine dashed line), nucleic acid (A_{260}, thick dashed line), and PAS-stained materials (solid circle); (B, D), RGM21- (open square) and HIK1083- (solid square) reactive materials. The fractions indicated by the bar were pooled and used for further characterizations.

under reduced conditions, most PAS-stained macromolecules were slightly retained on the column (Fig. 1C). Detection with MAbs revealed specific elution patterns for the extracts; HIK1083 detected the excluded fraction, while RGM21 recognized fractions which were slightly included (Fig. 1D). Thus, the results indicated the presence of at least two mucin species of different sizes in the corpus of the rat stomach. Because of the incomplete separation of these mucin species, they were applied together for further purification.

Figure 2 shows the results of CsTFA equilibrium centrifugation. Similar profiles were obtained from the non-reduced and reduced macromolecules. Mucins detected by the phenol/H_2SO_4 method or PAS were recovered at a density of 1.36 to 1.45 g/ml, and they were completely separated from nucleic acids as described (*23*); nucleic acids were detected at a density of 1.57 by absorbance at 260 nm and also by the phenol/H_2SO_4 method. RGM21-reactive mucins showed a similar profile to those detected by the phenol/H_2SO_4 method and to the profile of PAS-reactive mucins. In contrast, HIK1083-reactive mucins were recovered at peak density of 1.40 in a relatively narrow range.

Effects of Disulfide Bond Reduction on Size of Mucins—To evaluate the effect of reduction of disulfide bonds, the purified whole mucins and subunits were subjected to Sepharose CL-2B chromatography. Whole mucins recognized by either RGM21 or HIK1083 were still not retained on the column (Fig. 3A). Most of the HIK1083-reactive subunits were also not retained on the column (Fig. 3B). In contrast, RGM21-reactive subunits were retained on the column. The results showed that RGM21-reactive mucins had structures multimerized by disulfide bonds. However, it is not clear whether HIK1083-reactive mucins have multimerized structures. To clarify this point, rate-zonal sedimentation centrifugation was performed. Figure 4 shows that the sedimentation velocity of both RGM21- and HIK1083-reactive mucins was decreased due to reduction of the disulfide bonds. The results indicated that both types of mucins had multimerized structures. Furthermore, the

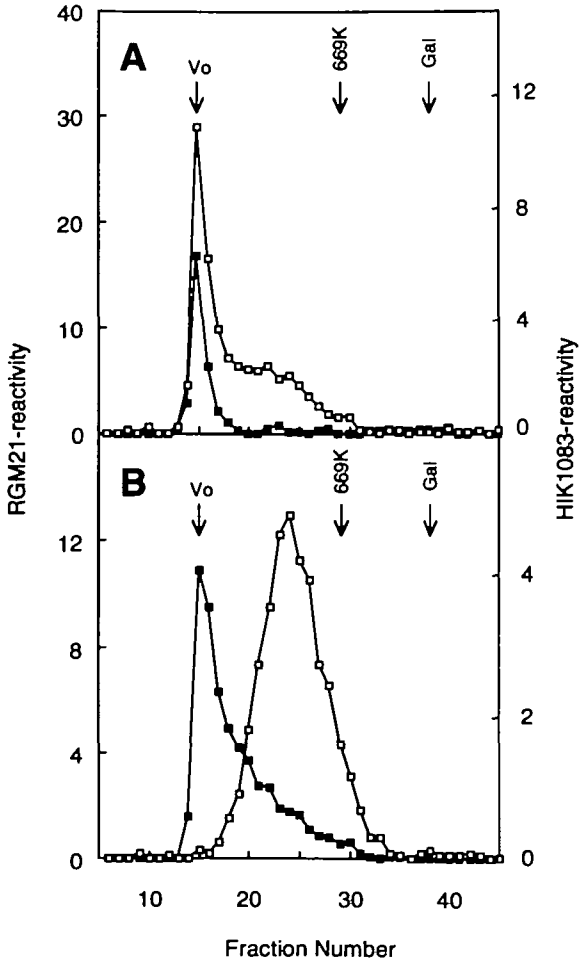

Fig. 3. **Sepharose CL-2B chromatography of the purified whole mucins (A) and subunits (B).** The purified mucins were loaded onto a column of Sepharose CL-2B equilibrated with 4 M GuHCl, pH 7.4, containing 0.5% Triton X-100, 50 mM Tris, 10 mM EDTA, 2 mM benzamidine hydrochloride, and 1 mM PMSF. Aliquots of each fraction were assayed for RGM21- (open square) and HIK1083- (closed square) reactive materials. The column was calibrated with bovine thyroglobulin (669K) and galactose (Gal).

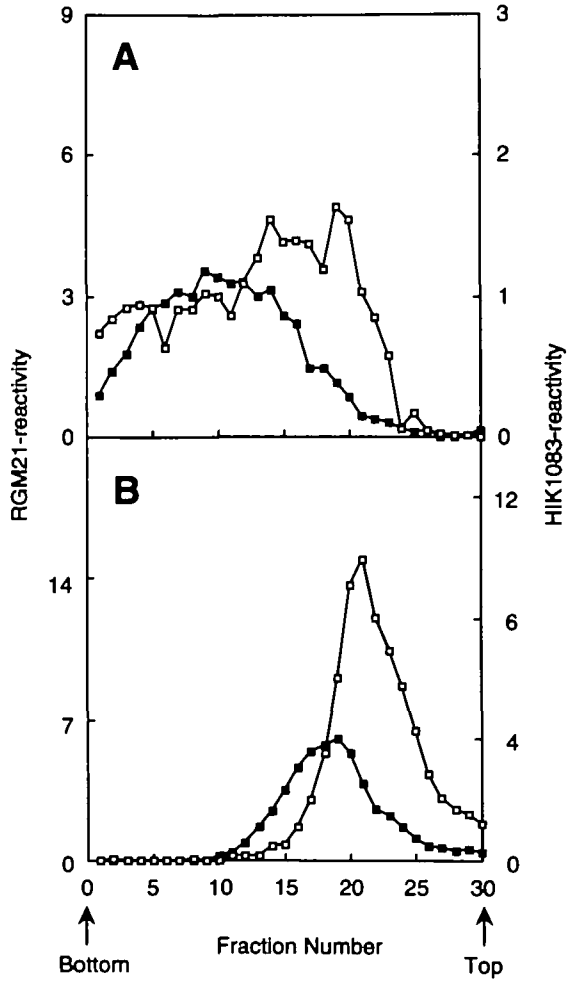

Fig. 4. **Rate-zonal sedimentation centrifugation of the purified mucins.** The purified whole mucins (A) or subunits (B) were layered on a linear gradient of glycerol and centrifuged at 68,000×*g* for 24 h at 19°C as described in "MATERIALS AND METHODS". Each fraction was assayed for RGM21- (open square) and HIK1083- (closed square) reactive materials.

results showed that the HIK1083-reactive mucin was larger than the RGM21-reactive mucin in whole mucins as well as in subunit form.

Separation of Mucin Subunits by Q-Sepharose Chromatography—The mucin subunits were subjected to Q-Sepharose chromatography. They were divided into at least four fractions, designated UB, B1, B2a, and B2b (Fig. 5). UB passed through the column under the test conditions, while the others were bonded to the column and eluted by the salt gradient. HIK1083-reactive subunits were mainly found in UB and slightly in B1. In contrast, RGM21-reactive subunits were recovered in B1, B2a, and B2b but not in UB. Dot-blot analyses showed that B2a and B2b were stained strongly with PAS, while UB and B1 were only moderately stained (Fig. 5A). B2b was also stained by HID, indicating that it contained sulfate residues, probably sulfated carbohydrate chains. The results showed that the subunits possessed different glycoforms.

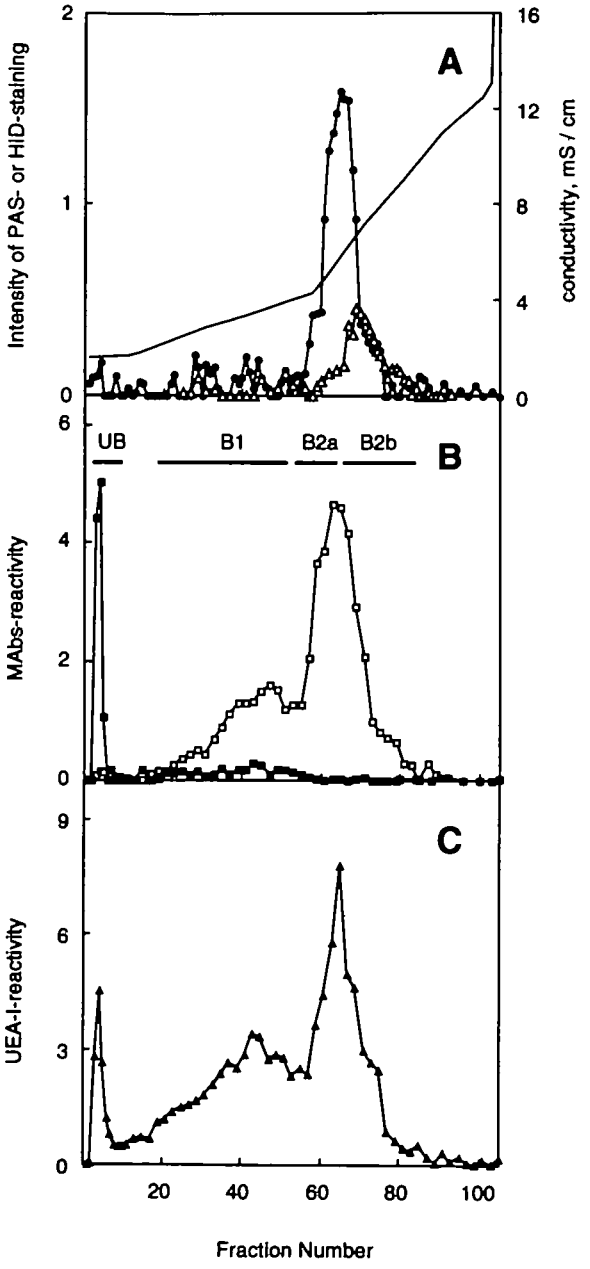

Fig. 5. **Q-Sepharose chromatography of the mucin subunits.** The mucin subunits were loaded onto a Q-Sepharose column equilibrated with 8 M urea containing 30 mM Tris-HCl, pH 7.4, and 0.5% Triton X-100 and eluted with the same solution followed by a salt-gradient. Each fraction was assayed for: (A), conductivity (straight line), and PAS- (solid circle), and HID- (open triangle) stained materials; (B), RGM21- (open square) and HIK1083- (solid square) reactive materials; (C), UEA-I-reactive materials (solid triangle).

To test whether mucins reacting with neither RGM21 nor HIK1083 are present, ELLA assay was performed. UEA-I was chosen as lectin for this assay because it reacted with mucosal mucins (*24*). Figure 5c shows that no mucin species were present that reacted with UEA-I but did not react with one of the MAbs.

Characterization of Mucin Subunits—To characterize the mucin subunits separated by Q-Sepharose chromatography further, they were subjected to Sepharose CL-2B chromatography. HIK1083-reactive UB passed through the column, as already described (data not shown). A small amount of HIK1083-reactive B1 also passed through from the column. RGM21-reactive B1, B2a, and B2b were eluted similarly to Fig. 3, but B1 was slightly retarded compared to B2a or B2b (data not shown).

Next, the amino acid and carbohydrate compositions of HIK1083-reactive UB and RGM21-reactive B1, B2a and B2b were determined. Table I shows that all subunits had typical amino acid compositions of mucin. However, there were some differences among the subunits. UB and B1 had a lower threonine and proline contents than B2a or B2b. The results indicate that UB and B1 may have core proteins different from those of B2a and B2b. Carbohydrate composition analysis showed that UB had less fucose than the

TABLE I. **Amino acid and carbohydrate composition of the mucin subunits.**

	UB	B1*	B2a	B2b
	Amino acid composition (residues/100 residues)			
Asx	6.6	7.9	7.4	8.6
Glx	12.8	14.4	11.5	12.6
Ser	11.7	13.5	13.2	13.2
Gly	17.0	18.7	13.7	14.5
His	0.8	0.6	1.2	1.1
Arg	4.1	4.3	3.9	3.7
Thr	5.4	6.1	10.9	9.5
Ala	5.3	4.4	4.9	5.2
Pro	3.3	4.0	6.8	5.9
Tyr	8.5	6.0	6.8	5.2
Val	4.3	4.3	4.8	4.9
Met	3.5	1.3	2.3	1.6
Ile	3.8	3.7	3.5	3.5
Leu	5.7	6.1	4.6	5.3
Phe	3.5	2.8	2.5	3.0
Lys	2.1	2.0	1.8	2.1
	Carbohydrate composition (GalN = 1.0)			
GalN	1.0	1.0	1.0	1.0
GlcN	2.4	2.9	3.4	3.2
Gal	2.2	3.3	2.2	4.8
Fuc	0.4	1.3	1.0	1.9
Neu5Ac	0.03	0.05	0.05	0.09
Protein content (wt%)	28	19	13	15

*RGM21-reactive B1 obtained by Sepharose CL-2B chromatography was assayed. Values are expressed as an average value of three experiments.

others. Furthermore, UB had less sialic acids than B2a or B2b, as expected from the elution position on ion-exchange chromatography. The results support the evidence from dot-blot analyses that each subunit separated by Q-Sepharose chromatography has a different glycoform. The protein content of UB was relatively high compared to the other fractions.

DISCUSSION

Recent studies showed that MUC1, MUC5AC, and MUC6 were components of human gastric mucin (*5-7*). Of these, MUC5AC and MUC6 are considered to be gel-forming mucins (*25*). MUC5AC was found in surface mucosa and MUC6 in glandular mucosa (*5-7*). Because a rat *MUC5AC* gene bearing homology to the human *MUC5AC* gene was reported (*26*), it is expected that rat MUC5AC and MUC6 are also present in the stomach. If this is the case, the mucin species present in the surface mucosa of the rat stomach might differ from those in the glandular mucosa.

The present study indicated that mucin subunits recognized by MAb RGM21 differ from those recognized by HIK1083 with respect to their density, size and charge. Because histochemical studies showed that RGM21 stained surface mucosa while HIK1083 stained glandular mucosa in the rat corpus (*13*), the two MAbs were expected to detect the surface mucins and glandular mucins separately. RGM21-reactive subunits, B1, B2a, and B2b, seem to be derived from the surface mucosa, and HIK1083-reactive UB from the glandular mucosa. Dot-blot analyses supported this idea. That is, B2a and B2b were stained strongly with PAS, while UB was faintly stained. This is consistent with the histochemical observation that the surface mucosa was stained strongly with PAS, while the glandular mucosa was lightly stained, although why B1 was stained only slightly by PAS was not explained. Thus, our data indicated that different mucin species were associated with the surface and glandular mucosa in the corpus of the rat stomach. Recently, Nordman *et al.* reported that surface and glandular mucin species in the pig stomach were different (*11*). Although glycoforms of the mucins might not be the same among animal species, different mucin species may generally be present in the surface and glandular mucosa.

At least two mucin core proteins, rat MUC5AC core protein and another one, may be expressed in surface mucosa of the rat corpus, because B1 seems not to have the same core protein as B2a or B2b based on their amino acid composition, although there are no data on which subunit corresponds to rat MUC5AC. In contrast, B2a and B2b seem to have the same core protein in spite of their different HID-staining intensity. It was reported that surface mucins in the corpus of the pig stomach consisted of several species of subunits, which may have the same core proteins (*10*). Further studies may be required to determine whether different core proteins are present in the surface mucosa of the rat stomach.

Byrd *et al.* reported that PCS-stained human gastric mucin was MUC6 (*27*). The staining pattern of HIK1083 and that of PCS were completely the same in both human and rat stomach (*14*), and our previous study showed that rat gastric mucins stained with PCS were excluded from the Sepharose CL-2B column (*9*), in the same way as the HIK1083-reactive mucins. Therefore, it is likely that the HIK1083-reactive mucin subunit, UB, is rat MUC6, although the sequence of rat MUC6 has not been reported.

The present study indicated that both RGM21- and HIK1083-reactive mucins had a multimerized structure. When two or more subunits are present, there are two possibilities in constructing a multimerized structure: one is that each subunit multimerized separately, and the other is that the subunits were multimerized together. HIK1083-reactive subunit UB seems to multimerize by itself, because the density of HIK1083-reactive whole mucins was quite similar to that of the subunits. However, the possibility that UB may have multimerized with other unknown subunits was not neglected. There are no data so far to conclude whether RGM21-reactive subunits, B1, B2a, and B2b, multimerized together. Q-Sepharose chromatography of the whole mucins may answer this question, but unfortunately this did not succeed because of very low recovery. Further study is required to determine whether they multimerized together.

Ota and Katsuyama reported that GOCTS-stained surface and PCS-stained glandular mucin species formed individual gel layers in the stomach surface (*4*). The distinct properties of the surface mucins from the glandular mucins described in this paper seem to contribute to construction of a separate gel layer. For example, due to their large size at the level of whole mucin and/or subunits, HIK1083-reactive mucins may have a specific gel-forming nature. Katsuyama and coworkers reported that *Helicobacter pylori*, the bacterium presumed to cause many gastric diseases, was observed in the surface mucous gel layer consisting of GOCTS-stained surface mucins but not in that consisting of PCS-stained glandular mucins in human gastric mucosa (*28*). A specific carbohydrate residue, such as sialic acid, has been reported to be involved in attaching of *Helicobacter pylori* to human gastric mucins (*29*). A distinct glycoform of the surface mucins may contribute to survival of the bacteria in the surface mucous gel layer. Further characterization of mucin species may reveal individual physiological functions of surface and glandular mucins in the gastric mucosa.

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