Biosynthesis of rat MUC2 in colon and its analogy with human MUC2

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In order to identify the mucins synthesized and secreted in the rat colon, we studied their biochemical characteristics and biosynthesis and evaluated their analogy to human colonic mucins. Purified mucin from both species appeared similar with respect to composition, buoyant density and mobility on SDS/PAGE. Isolated rat colonic mucin (RCM) was used to elicit a polyclonal antiserum, which was used in metabolic labelling studies to identify mucins and mucin precursors. RCM is synthesized as a 600 kDa precursor protein, which oligomerizes before O-glycosylation. The mature, high-molecular mass mucin is secreted

and displays an anomalous molecular mass on SDS/PAGE of approximately 650 kDa. Polymorphism in precursor size was found among different rats, suggesting genetic heterogeneity. Molecular mass, biosynthesis and secretion of RCM appeared similar to human MUC2. Moreover, RCM precursor could be immunoprecipitated using specific anti-(human MUC2) antisera, indicating that the RCM can be designated rat MUC2. This study describes the biosynthesis of two homologous mucins in two different species. The high degree of similarity suggests functional analogy.

INTRODUCTION

Secretory mucins are the major constituents of the protective mucus layer which covers many epithelia. Mucins often mediate interactions between epithelial cells and their environment. It is thought that alterations in the structure or amount of mucins may affect these protective and interactive functions. Therefore, mucins may play a role in diseases in which the epithelium is chronically inflamed, as in inflammatory bowel disease. Mucins generally consist of a core protein containing tandemly repeated amino acid sequences rich in serine and threonine residues, which are heavily O-glycosylated. The C- and N-termini of the protein protrude from the heavily glycosylated central region and contain non-repetitive amino acid sequences. Cysteine residues are present in the C- and N-termini and are likely to play a role in the formation of both intra- and inter-molecular disulphide bridges. The protruding protein parts are less glycosylated, containing some N-glycosylation, rendering them susceptible to proteolytic breakdown, in contrast to the central glycopeptide which is proteinase resistant [1–3].

For rat, at least four partial mucin cDNAs have been cloned from cDNA libraries [4–8]. Moreover, human MUC1 cDNA probe has been shown to hybridize to rat genomic DNA, suggesting that a fifth mucin, homologous to human MUC1, also exists in rat [9]. In rat intestine, partial cDNAs of two different mucins were detected, designated mucin-like peptide (MLP) and RMUC 176 [5,7,8,10–14]. The cDNA of MLP was isolated from small intestine and showed degenerate tandem repeats of 11–12 amino acids, rich in serine, threonine and proline residues [8,13]. These degenerate repeats showed some amino acid sequence homology with human MUC2, composed of short sequences of 3–6 amino acids. However, in the C-terminal part of MLP, comprising a 90 kDa polypeptide, 73% homology was found with human MUC2 C-terminus [7,10,11]. Moreover, a cDNA nucleotide sequence, recently isolated from a rat intestinal cDNA library, encoded an N-terminus showing 80% homology with the human MUC2 N-terminal, and was

designated rat MUC2 [15]. Whether the cDNAs of MLP and rat MUC2 are part of the same gene, has still to be confirmed. RMUC 176 is a rat cDNA clone which can be distinguished from MLP by Northern blotting [5,7]. RMUC 176 contains a large number of nearly identical 6-amino acid repeats, and a small non-repeated sequence which shows no homology to any known mucin [5,7,16]. To date, no biosynthetic studies on rat colonic mucin (RCM) have been published.

Of the seven human mucin genes identified so far, six have been reported to be expressed, to some extent, in the colon [16–22]. Of these mucins, MUC2 and MUC3 were isolated from human intestinal cDNA libraries [16,17]. We have recently described the biosynthesis of human MUC2, which is the predominant mucin synthesized in the human colon [23]. In this study, we isolated and analysed RCM and studied its biosynthesis. By comparing RCM with human colonic mucin (HCM) we identified a broad resemblance in structure as well as biosynthesis, suggesting that the colonic mucins from human and rat may be functionally analogous.

MATERIALS AND METHODS

Unless otherwise indicated, chemicals were obtained from the following manufacturers: Gibco-BRL, Gaithersburg, MD, U.S.A.; Merck, Darmstadt, Germany; Sigma, St Louis, MO, U.S.A.; BioRad, Richmond, CA, U.S.A.; Pharmacia, Uppsala, Sweden; BDH, Poole, Dorset, U.K.; Boehringer, Mannheim, Germany; and Amersham, Buckinghamshire, U.K.

Isolation of RCM

The colons of 30 Wistar rats were dissected, cut open along their lengths and rinsed in cold PBS. The mucosae were scraped. Total mucin was isolated from scrapings as was previously described for rat gastric mucin and HCM [23,24]. Briefly, scrapings were homogenized for 20 h at 4 °C in homogenization buffer, containing 6 M guanidinium-HCl (Sigma) and 100 mM dithiothreitol (DTT; Sigma) for reduction in order to enhance solubility.

Abbreviations used: MLP, mucin-like peptide; RCM, rat colonic mucin; HCM, human colonic mucin; anti-RCM, anti-rat-colonic-mucin antiserum; anti-HCM, anti-human-colonic-mucin antiserum; DTT, dithiothreitol; PAS, periodic acid/Schiff's reagent; endo H, endo- β -N-acetylglucosaminidase H; RER, rough endoplasmic reticulum.

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Sulphydride groups were carboxymethylated through addition of 250 mM iodoacetamide (Sigma) and stirring for another 24 h at 4 °C. Mucins were purified by equilibrium centrifugation on triple CsCl (Boehringer) density gradients, 70 h at 4 °C. The first gradient had a guanidine hydrochloride concentration of 4 M with CsCl added to a density of 1.40 g/ml. Mucin-containing fractions were pooled and run on an identical second gradient. For the third gradient, CsCl was added to a density of 1.50 g/ml to the pooled mucin-containing fractions in guanidine hydrochloride at a concentration of 0.2 M, to separate mucin from nucleic acids [25].

Analysis of RCM

The boyant density of fractions was measured by weighing 1 ml of each fraction. Dialysed samples from fractions were analysed by SDS/PAGE [26]. Unless otherwise indicated, all gels were composed of 3 % stacking gel/4 % running gel, prepared from a stock solution containing 30 % acrylamide (Gibco) and 0.8 % piperazine diacrylamide (BioRad). All samples were boiled for 5 min in sample buffer containing 1 % (v/v) 2-mercaptoethanol (BioRad) and 1 % (w/v) SDS (BDH). Prior to SDS/PAGE, some samples were treated with 0.1 mg/ml proteinase K (Merck) or 10 mg/ml DNase I (Sigma) or 10 mg/ml RNase A (Sigma), in 50 mM Tris pH 7.4, and additionally with 1 mM MgCl₂ (Merck) in the case of DNase I, for 1 h at 37 °C, before analysis by SDS/PAGE. Prestained high molecular mass markers from BioRad with largest molecular mass of 205 kDa were used, and in addition, [³⁵S]amino acid-labelled, unreduced rat gastric mucin precursor was used for reference to molecular mass of approx. 300 and 600 kDa [27]. Proteins in gels were stained with Coomassie Brilliant Blue R-250 (Merck), carbohydrates with periodic acid/Schiff's reagent (PAS; Sigma) [28] and nucleic acids and glycoproteins with silver staining (BioRad) according to the manufacturers' protocols. Hexose was quantitated in an orcinol (Sigma) assay at A₅₄₀ [29]. Monosaccharide analysis was carried out according to the method of Savage et al. [30]. Amino acid analysis was performed using the *O*-phthalaldehyde (Pierce, Oud Beijerland, The Netherlands) derivative technique and HPLC [31].

Preparation of polyclonal antiserum against RCM

Purified antigen (1 ml) containing about 500 µg of RCM was mixed with 1 ml of Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.) and injected subcutaneously into a New Zealand White rabbit. The rabbit was boosted on day 14 and day 21 with an identical aliquot of RCM mixed with 250 µl of Freund's incomplete adjuvant (Difco). Serum was obtained from blood collected on day 27.

Characterization of the antiserum against RCM

To study the reactivity of the anti-RCM antiserum (anti-RCM), Western blotting and immunohistochemistry were performed as previously described [23]. HCM and anti-HCM antiserum (anti-HCM) were obtained as described previously [23]. Briefly, purified RCM and HCM were run on SDS/PAGE and then blotted onto nitrocellulose (Nitran; Schleicher & Schuell, Germany). Nitrocellulose blots were incubated with anti-RCM or anti-HCM, both diluted 1:500 in blotbuffer containing 5 % non-fat milk powder (Lyempf, Kampen, The Netherlands). Thereafter, the nitrocellulose blot was incubated with goat anti-(rabbit IgG) conjugated to horseradish peroxidase (BioRad) and stained with 500 mg/l 3,3'-diaminobenzidine (Sigma). Reactivity towards peptide epitopes was tested by digesting the peptide

epitopes of RCM and HCM before SDS/PAGE with 0.1 mg/ml proteinase K (Merck) as described above. Reactivity towards carbohydrate epitopes was tested by oxidizing oligosaccharides of blotted RCM using 5 % (w/v) periodic acid (Sigma) for 1 h at 4 °C and stopping this reaction with 2 mg/ml sodium borohydrate (Merck) for 15 min at room temperature, before incubation with the antisera. As a control for oxidation, incubation with an anti-bovine gall bladder mucin antiserum was used, which was known to cross-react with carbohydrate epitopes of RCM [32]. Immunohistochemistry was performed as previously described [33]. Briefly, rat colon was dissected and embedded in paraffin. Consecutive sections of 7 µm were incubated with 1:500 anti-RCM or 1:1000 anti-HCM, and then with goat anti-(rabbit IgG) conjugated to horseradish peroxidase and 3,3'-diaminobenzidine.

Metabolic labelling studies

Rat colon was dissected, rinsed with PBS and cut into pieces of 4 × 4 mm and placed in glass vials in 1 ml of Eagle's minimum essential medium (Gibco-BRL) supplemented with non-essential amino acids (Sigma) (0.1 mM per amino acid), 100 U/ml penicillin/streptomycin (Sigma) and 2 mM glutamine (Sigma) at 37 °C under 95 % O₂/5 % CO₂. For comparison with HCM, human colonic biopsies were obtained at endoscopy from patients without colonic disease and the same protocol for metabolic labelling was followed. Biosynthesis was studied as previously described [23,24]. Briefly, to study the biosynthesis of RCM polypeptide, the tissue was first depleted of methionine and cysteine. Then [³⁵S]amino acids (Cell Labelling Mix, specific activity 1000 Ci/mmol; Amersham), containing about 65 % L-[³⁵S]methionine and about 25 % L-[³⁵S]cysteine, were added to the medium. Alternatively, explants were labelled with radioactive sulphate, which is incorporated into mature mucins [27]. Therefore, the tissue was depleted of sulphate. Then [³⁵S]sulphate (specific activity 1050 Ci/mmol; Amersham) was added. Glycosylation was studied, by adding D-[1-³H]galactose (specific activity 20 Ci/mmol; Amersham) to the medium. In general, pulse-labelling was performed at 1 mCi radiolabel per ml. Chase-incubations were performed by exchanging the medium with the above-mentioned radioactive compounds, for medium containing 0.5 mM L-methionine (Sigma), 0.1 mM L-cysteine (Merck) and 200 mg/l magnesium sulphate (Merck). Tissue samples were homogenized in buffer, containing 1 mM phenylmethanesulphonyl fluoride, 100 µg/ml pepstatin A (Sigma), 100 µg/ml leupeptin (Sigma), 10 mM iodoacetamide (Sigma), 1 mg/ml soybean trypsin inhibitor (Sigma) and 0.24 U/ml aprotinin (Sigma). Mucin was immunoprecipitated from the homogenates with anti-RCM or with anti-HCM. Anti-HCM is a polyclonal antiserum, elicited against purified HCM, which was shown to react with the protruding polypeptide parts of human MUC2 [23]. Alternatively, either the monoclonal antibody WE9 was used [34], which recognizes RCM in a similar manner to anti-RCM, or a specific anti-MUC2 antiserum was used, directed against human MUC2 amino acid repeats [22]. Immune-complexes were precipitated using either Sepharose CL-4B-coupled protein A (Pharmacia) or fixed *Staphylococcus aureus* (IgGSorb, Enzyme Center, Malden, MA, U.S.A.). Immuno-precipitated mucins were spun down and washed several times. To be able to distinguish between precursor proteins and mature fully glycosylated RCM, some immunoprecipitated samples were treated with 750 U endo-β-N-acetylglucosaminidase H (endo H; New England Biolabs, Beverly, MA, U.S.A.) as described earlier [23,24,35]. Samples were run on SDS/PAGE, gels were stained with PAS and radiolabelled mucins were analysed by

fluorography of the gel. Alternatively, radiolabelled mucins were analysed by gel filtration, essentially as described for rat gastric mucin [36,37]. [35 S]Sulphate-labelled and immunoprecipitated RCM was run on a 100 $\times$ 1.6 cm Sephacryl S-1000 column (Pharmacia) in buffer containing 4 M guanidine hydrochloride, 50 mM Tris, pH 7.5, and 5 mM EDTA. Fractions of 9 ml were collected and radioactivity was counted in a liquid-scintillation counter. For some experiments, [3 H]galactose-labelled mucin was electro-eluted overnight from SDS/PAGE gel-slices, using the Model 422 Electro-Eluter Assembly (BioRad), in buffer containing 25 mM Tris, 192 mM glycine and 0.1 % SDS.

RESULTS

Isolation of RCM

RCMs were isolated using triple CsCl density gradients. Figure 1 shows the result of the final gradient. Fraction numbers 5–8 of the third gradient had a mean density of 1.50 g/ml, corresponding with a peak in the hexose assay (Figure 1a). SDS/PAGE analysis, followed by PAS staining, showed one high-molecular mass band, while Coomassie Blue staining of the same gel showed no contamination with proteins (Figure 1b). Silver staining of similar gels, containing samples of each fraction from the final gradient, treated with either DNase I, RNase A or proteinase K, showed that DNA was present in fractions 1–3, while RNase A- or proteinase K-sensitive material was not detected in any fraction (results not shown). Fractions 5–8, containing purified RCM, were pooled and dialysed. Purification of mucin from 1.23 g of

Figure 1 Isolation of RCM

(a) Graphic representation of density (■) and orcinol assay (▲) of fractions of the final CsCl density gradient. (b) 4% stacking gel/7.5% running gel SDS/PAGE, containing samples of fractions 1–12 as in (a). Gel was stained with both PAS and Coomassie Blue. Fractions 5–8 were pooled and designated RCM. The arrow indicates the border between the 4% stacking and the 7.5% running gels. Prestained high-molecular mass markers (in kDa) are indicated on the left.

Figure 2 Influence of SDS on mobility of RCM and proteinase K-digested RCM on PAGE

RCM and proteinase K-digested RCM were run on 4% stacking gel/7.5% running gel PAGE, with and without SDS: —, indicates that neither the samples nor the gel contained SDS, and gels were stained with PAS. The arrowhead indicates the border between the 4% stacking and the 7.5% running gels. Prestained high-molecular mass markers are indicated on the left.

Table 1 Amino acid and carbohydrate analysis of RCM and HCM

Composition of RCM was determined in the pooled fractions containing purified mucin as displayed in Figure 1. The composition of HCM was previously determined [23]. * Present in small amounts but data are conflicting; N.d., not determined.

Amino acid	Content (mol %)		Carbohydrate	Content (mol %)	
	RCM	HCM		RCM	HCM
Asp	5.5	5.4	Fucose	6.2	5.0
Thr	32.5	38.3	Mannose	0.1	2.1
Ser	14.4	8.0	Galactose	24.4	16.9
Glu	6.3	6.4	GalNAc	21.3	12.4
Pro	15.5	16.1	GlcNAc	40.1	44.2
Gly	7.9	11.5	NeuAc	7.6	19.6
Ala	1.7	1.9			
Cys	N.d.	N.d.			
Val	3.4	4.5			
Met	> 0*	> 0*			
Ile	3.4	1.7			
Leu	3.3	3.6			
Tyr	1.8	0.6			
Phe	> 0*	> 0*			
His	0.6	0.7			
Lys	2.2	1.4			
Arg	> 0*	> 0*			

wet weight of mucosal scrapings yielded approximately 1.8 mg of purified RCM.

The influence of SDS on the mobility of RCM and proteinase K-digested RCM on PAGE was tested by electrophoresis in the presence or absence of SDS (Figure 2). This showed very similar mobilities of the high-molecular mass PAS-stained bands of RCM and proteinase K-digested RCM, indicating that the mobility of RCM on gel was independent of saturation with SDS.

Amino acid and carbohydrate composition were analysed and compared with HCM, which was isolated previously (Table 1). Amino acid analysis of RCM revealed a distribution that is characteristic of mucins, with high amounts of serine, threonine and proline residues. The carbohydrate composition of RCM

Figure 3 Epitope specificity of anti-RCM

In all panels: lane 1, RCM; lane 2, proteinase K-digested RCM; lane 3, HCM; lane 4, proteinase K-digested HCM. Panel (a), PAS staining of 3% stacking gel/4% running gel SDS/PAGE. Panels (b-d), Western blots from identical gels as in panel A, incubated with 1:500 anti-RCM (Panel b), with 1:500 anti-HCM (Panel c) or with 1:500 anti-RCM after treatment of the blot with 5% periodic acid (Panel d). The arrow indicates the border between the 3% stacking and the 4% running gels. The prestained high-molecular mass marker of 205 kDa is indicated on the left. The positions of the unreduced rat gastric mucin precursors (300 and 600 kDa) are also indicated on the left, as revealed by autoradiography of Western-blots containing these markers.

was also characteristic for mucin, showing *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, fucose, *N*-acetylneuraminic acid and mannose.

Characterization of the anti-RCM

Figure 3(a) shows RCM and HCM and proteinase K-digested RCM and HCM visualized on SDS/PAGE by PAS staining. RCM is visible as one band with very low mobility on SDS/PAGE, comparable to HCM. Proteinase K-digestion resulted in only a slight increase in the mobility of RCM as well as HCM, while the intensity of the PAS staining remained unaffected. Most likely, proteinase K removed only the peripheral parts of the polypeptides while the large central heavily O-glycosylated glycopeptide remained intact. Identical gels were used for Western blotting. Anti-RCM recognized both RCM and HCM, indicating the presence of identical epitopes on both mucins (Figure 3b). Moreover, it also recognized a band of slightly higher mobility than RCM. This band became more prominent in the RCM-preparations after repeated freezing and thawing and may thus represent a degradation product of RCM. After proteinase K digestion, no recognition of either proteinase K-digested RCM or of proteinase K-digested HCM, nor of the band of slightly higher mobility, could be detected. Therefore, anti-RCM most likely recognized similar peptide epitopes on HCM and RCM. Cross-reactivity of anti-HCM towards RCM, but not towards proteinase K-digested RCM, can be seen in Figure 3(c). In rat colonic mucosal homogenate, mucin could be visualized on SDS/PAGE by PAS staining as one major band, with similar mobility to RCM on SDS/PAGE (results not shown). Anti-RCM as well as anti-HCM recognized a similar band on Western blot of rat colonic mucosal homogenate, while reactivity was lost after proteinase K digestion (results not shown). After treatment of HCM and RCM with periodic acid, some loss of recognition was seen, indicating that anti-RCM also reacted to some extent with carbohydrate epitopes (Figure 3d). Reactivity of anti-gall-bladder mucin antiserum, which was known to cross-react with carbohydrate epitopes of RCM, was completely lost after periodic acid oxidation, indicating that the

Figure 4 Histochemistry of rat colonic mucosa

Panel A, haematoxylin and azofoxin staining of a tissue section from normal distal rat colon. Panel B, immunohistochemistry of an adjacent tissue section after incubation with 1:500 anti-RCM. Panel C, immunohistochemistry of an adjacent tissue section after incubation with 1:1000 anti-HCM. Panel D, control immunohistochemistry of an adjacent tissue section where the first antiserum was exchanged for PBS. S, serosa; L, lumen. Scale bar indicates 100 μ m.

periodic acid treatment was sufficient to oxidize carbohydrate epitopes (results not shown).

Recognition of mucins by anti-RCM and anti-HCM was also studied histologically. Figure 4 shows the immunohistochemistry of adjacent sections of rat distal colon. The morphology of normal rat colon is shown in Figure 4(a). When a similar section was incubated with anti-RCM (Figure 4b), the apical part of all goblet cells, containing the mucin granules, were stained. Throughout the section, staining of the goblet cells appeared heterogeneous, a phenomenon for which we have no unequivocal explanation. As mucin granules are notoriously difficult to fix, this heterogeneity may be introduced by fixation artifacts. On the other hand, the goblet cells may contain different amounts of the RCM detected by anti-RCM. Resolution of the staining did not permit any conclusion on the staining of rough endoplasmic reticulum (RER) or Golgi apparatus. Using anti-HCM, similar staining of rat colonic goblet cells was seen (Figure 4c). No reactivity was detected in the negative control, indicating that staining of goblet cells by either anti-RCM or anti-HCM was specific (Figure 4d).

Biosynthesis of RCM

As anti-RCM recognizes primarily the protruding, less-

Figure 5 Biosynthesis of RCM

Adjacent explants from rat distal colon were pulse-labelled with [³⁵S]amino acids ([³⁵S]AA) or with [³⁵S]sulphate (³⁵SO₄²⁻) for 30 min and chase-incubated for 4 h. RCM was immunoprecipitated using anti-RCM and divided into two identical aliquots, one of which was treated with endo H while the other served as control, before analysis on 3% stacking gel/4% running gel SDS/PAGE. In all panels: 1, pulse-incubation 30 min; 2, chase-incubation 4 h; 3, medium after 4 h chase-incubation. Endo H indicates incubation with endo H. (a) Fluorograph of pulse-chase experiment. (b) PAS staining of the SDS/PAGE gel containing the samples displayed in (a). The arrow indicates the border between the 3% stacking and the 4% running gels. Molecular mass markers are indicated on the left, i.e. unreduced rat gastric mucin precursor (600 and 300 kDa) and a prestained high-molecular mass marker (205 kDa).

glycosylated parts of the protein, it should be able to immunoprecipitate mucin precursor as well as fully O-glycosylated mature mucin, irrespective of their extent of O-glycosylation. After metabolic labelling, mucins were immunoprecipitated from explant homogenates and media using anti-RCM. The specificity of anti-RCM was tested by immunoprecipitating radiolabelled RCM, using aliquots of anti-RCM previously incubated with purified unlabelled RCM. Using this saturated aliquot of anti-RCM, a strong decrease in immunoprecipitated radiolabelled bands was observed, indicating the specificity of anti-RCM (results not shown).

After 30 min of pulse-labelling with [³⁵S]amino acids, RCM was visible as one band, which, compared with unreduced rat gastric mucin, corresponded to an apparent molecular mass of about 600 kDa (Figure 5a). This band showed a slightly higher mobility after endo H treatment, indicating that high mannose N-linked glycans were cleaved off. This indicates that the band represents a glycoprotein present in the RER. This component was the earliest detectable RCM precursor, as pulse-labelling for only 15 min also yielded this one band (results not shown). However, most experiments were performed using a 30 min pulse-labelling, as a 15 min pulse-labelling required much longer fluorographic exposure times. After 4 h of chase-incubation, this

endo H-sensitive '600 kDa' band could still be immunoprecipitated and, at the same time, a second band appeared with an estimated molecular mass of 650 kDa which was not susceptible to endo H-treatment. The '650 kDa' band, but not the '600 kDa' band, was secreted into the medium, and this secreted product was also not endo H sensitive. The time-dependent relationship between the '600 kDa' and '650 kDa' band suggests a precursor-product relationship between these glycoproteins. The endo H resistance, secretion and time-course of the appearance of the '650 kDa' product indicate that this band represents the mature mucin.

Figure 5(a) also shows immunoprecipitation of RCM after labelling with [³⁵S]sulphate. One band, with an apparent molecular mass of 650 kDa, was immunoprecipitated after 30 min of pulse-labelling, after 4 h of chase-incubation, as well as from the medium. No change in the mobility of the [³⁵S]sulphate-labelled band was observed during this pulse-chase experiment. This band was not sensitive to endo H treatment, further suggesting that it represents the mature mucin.

The immunoprecipitations displayed in Figure 5 appeared not to be completely quantitative, due to experimental constraints on the amount of anti-RCM that could be used without overloading the lanes of the gel. Therefore, the results should be regarded as a qualitative rather than a quantitative representation of the colonic RCM biosynthesis.

Immunoprecipitation of mucin with anti-RCM after [³H]galactose labelling showed results similar to [³⁵S]sulphate labelling (results not shown). Figure 5(b) shows the PAS staining of the gel corresponding with the fluorograph in Figure 5(a). Immunoprecipitation with anti-RCM from tissue homogenates and media yielded enough mature mucin to enable PAS staining; one PAS-stained band was immunoprecipitated, which coincided in position as well as band morphology with the '650 kDa' radiolabelled band detected in Figure 5(a). Moreover, like the radiolabelled '650 kDa' band, the PAS-stained band was not sensitive to endo H deglycosylation. Thus, the PAS-stained band offers an internal reference for the mobility of the mature mucin in all lanes, as exemplified in Figures 5(a) and 5(b). In [³⁵S]sulphate-labelled and PAS-stained rat colonic homogenates, the '650 kDa' band was always the only band visible on SDS/PAGE, indicating the high abundance of RCM in colonic homogenates (results not shown).

The difference in mobility on SDS/PAGE between the '600 kDa' and the '650 kDa' bands appeared small but significant. The subtle difference was detected in all 30 rats thus far examined by metabolic labelling studies as described above. It appeared that the radiolabelled '650 kDa' band always comigrated with the PAS-stained band, while the '600 kDa' [³⁵S]amino acid-labelled band could clearly be discerned from the PAS-stained band. On this point, it should be noted that the exact mobility of the '650 kDa' band varied from one gel to another. Apparently, subtle differences in preparation or running of the gel induce slight mobility changes in this anomalous migrating component. Most often, the '650 kDa' band migrates more slowly than the RCM precursor with an apparent molecular mass of 600 kDa, but occasionally the mature mucin migrates slightly faster than this precursor band. Therefore, we prefer to refer to this band as '650 kDa' to indicate that we do not wish to imply that this is the actual molecular mass of mature RCM.

RCM forms covalent oligomers

Secretory mucins are generally thought to form oligomers, essential for the gel-forming properties of these mucins [1]. To study oligomerization of RCM, this mucin was radiolabelled,

Figure 6 Oligomerization of RCM during biosynthesis

Rat colonic mucosa was pulse-labelled with [35 S]amino acids ([35 S]AA) for 30 min (p) and chase-incubated for 1 h (C1) and 4 h (C4). RCM was immunoprecipitated using anti-RCM, and analysed on non-reducing 3% stacking gel/4% running gel SDS/PAGE. Adjacent segments were labelled for 1 h with [35 S]sulphate ($^{35}\text{SO}_4^{2-}$), RCM was immunoprecipitated using anti-RCM and divided into two aliquots, one of which was reduced using 2-mercaptoethanol (2-ME) and the other which was unreduced. Furthermore, the PAS staining (PAS) is shown of the lanes containing the [35 S]sulphate-labelled samples, which are displayed as a fluorograph in the adjacent panel. Molecular mass markers (unreduced rat gastric mucin-precursor, in kDa) are shown on the left. Arrowheads 1 and 2 indicate the immunoprecipitated high-molecular mass products discussed in the text.

immunoprecipitated and analysed on non-reducing SDS/PAGE (Figure 6). After 30 min of pulse-labelling with [35 S]amino acids, RCM appeared as a band of approx. 600 kDa and a high-molecular mass band (band 1, Figure 6). During 1 h of chase-incubation, the 600 kDa band disappeared and RCM was visible as band 1 and as a band of even higher molecular mass (band 2, Figure 6). After 4 h of chase-incubation RCM was only visible as band 2. Unreduced, [35 S]sulphate-labelled mature RCM barely entered the 4% running gel while a substantial amount of mucin remained on top of the stacking gel (Figure 6). After reduction, all [35 S]sulphate-labelled mucin, which was localized on top of the stacking gel as well as the running gel, shifted to an apparent molecular mass of about 650 kDa. PAS staining of the gel showed a similar shift in mobility upon reduction, from the top of the running and stacking gel to approx. 650 kDa (Figure 6). Together, this indicates, that RCM is synthesized as a precursor protein of approximately 600 kDa, which over time forms disulphide-linked oligomers of increasingly high molecular masses (bands 1 and 2, Figure 6) which are subsequently glycosylated and sulphated.

Oligomerization was also studied on gel filtration. RCM was metabolically labelled for 3 h with [35 S]sulphate, immunoprecipitated with anti-RCM and half of the immune-isolated RCM was reduced by DTT. When non-reduced and reduced RCM were analysed on a Sephacryl S-1000 column, unreduced RCM eluted in the void volume, while reduced RCM eluted in the included volume (Figure 7). This indicated independently that mature RCM consists of disulphide-linked oligomers. Furthermore, it should be noted that RCM has a very high molecular mass, as reduced monomeric RCM could only be visualized in the included volume upon Sephacryl S-1000 gel filtration.

Identification of RCM

The RCM precursor protein from rat colon was compared with the HCM precursor protein synthesized in human colon. Rat colonic explants and human sigmoidal biopsies were both labelled with [35 S]amino acids, and mucins from homogenates of these

Figure 7 Oligomerization of mature RCM demonstrated on Sephacryl S-1000 gel filtration

Rat colonic explants were labelled for 3 h with [35 S]sulphate, RCM was immunoprecipitated using anti-RCM, and immune-isolated RCM was divided into two aliquots. One aliquot was reduced by 20 h of incubation at 4 °C with 100 mM DTT, the other aliquot was applied unreduced. The void volume and the total volume of the column corresponded to fractions 24 and 63 respectively. □, Unreduced [35 S]sulphate-labelled RCM; ■, reduced [35 S]sulphate-labelled RCM.

tissues were immunoprecipitated with either anti-RCM, anti-HCM, WE9 or anti-MUC2 (Figure 8). RCM precursor as well as HCM precursor could be immunoprecipitated using each of these antisera. The precursors of RCM and HCM show comparable apparent molecular masses on SDS/PAGE (Figure 8). Labelling of similar explants with [35 S]sulphate also showed similar cross-reactivity towards mature RCM and HCM, i.e. mature RCM as well as mature HCM were immunoprecipitated by anti-RCM, anti-HCM and WE9 (results not shown). The monoclonal antibody WE9 was raised against purified HCM [34] and recognized RCM and its precursor equally well, strongly suggesting recognition of a peptide epitope. Thus, the immunoprecipitation of both RCM precursor and HCM precursor by WE9 also indicates that similarity exists in the amino acid sequences of these mucins. Immunoprecipitation of HCM with

Figure 8 Identification of the RCM

Distal rat colonic explants were labelled for 30 min (R) and human sigmoidal biopsies for 45 min (H) with [35 S]amino acids. Mucins were immunoprecipitated from homogenates of these tissues using anti-RCM, anti-HCM, WE9 or anti-MUC2. The arrow indicates the border between the 3% stacking and the 4% running gels. On the left molecular mass markers are indicated in kDa (unreduced rat gastric mucin precursor).

Figure 9 RCM shows polymorphic expression among individual rats

Explants from ten individual rat colonic mucosae (lanes 1–10) were pulse-labelled for 30 min with [35 S]amino acids and chase-incubated for 4 h. The homogenates of pulse-labelled explants were divided in two, one aliquot was immunoprecipitated using anti-RCM (a), the other aliquot using WE9 (b). RCM was immunoprecipitated from homogenates after 4 h chase-incubation using anti-RCM (c). (d) PAS staining of the gel containing the radiolabelled samples displayed in (a). The arrow indicates the border between the 3% stacking and the 4% running gels. On the left molecular mass markers are indicated in kDa (unreduced rat gastric mucin precursor).

the specific anti-MUC2 identified HCM as human MUC2 [23]. As the RCM precursor was immunoprecipitated by anti-HCM as well as anti-MUC2, we can deduce that RCM is the MUC2 analogue in rat (Figure 8).

Local and individual differences in RCM biosynthesis

As mucins are known to display genetic polymorphism, variation in RCM biosynthesis in ten individual Wistar rats was studied. Colonic segments were labelled with [35 S]amino acids, chase-incubated for 4 h, and RCM was immunoprecipitated from homogenates using either anti-RCM or the monoclonal antibody WE9. The RCM precursors varied in apparent molecular mass between approx. 550 and 650 kDa, independent of the antibody used (Figures 9a and 9b). Moreover, some rats showed biosynthesis of two precursors of different molecular masses (e.g. rats 1 and 6, Figures 9a and 9b), which remained equally intense after chase-incubation (Figure 9c). The precursor heterogeneity was not reflected in the apparent molecular mass of the mature RCM, as detected by PAS staining, which showed similar bands

Figure 10 Biosynthesis of RCM along the proximal-to-distal axis of rat colon

The colon of one rat was cut into six segments of equal length, numbered 1–6, from proximal (P) to distal (D). Each of these segments was divided into two parts, one of which was labelled for 30 min with [35 S]amino acids (a), while the other was labelled with [35 S]sulphate (b). RCM was immunoprecipitated from tissue homogenates using anti-RCM. (c) PAS staining of gel containing the [35 S]sulphate-labelled samples displayed in (b). The arrow indicates the border between the 3% stacking and the 4% running gels. On the left molecular mass markers are indicated in kDa (unreduced rat gastric mucin precursor).

with apparent molecular mass of 600 kDa for all rats (Figure 9d).

Finally, we studied RCM biosynthesis along the longitudinal axis in rat colon in order to analyse possible regional differences in RCM expression or processing. After [35 S]amino acid-labelling, the RCM precursor was visible as a band with an apparent molecular mass of 600 kDa band in all segments of the colon (Figure 10a). We concluded that the RCM precursor displays the same molecular mass in the entire colon and that the precursor is synthesized in similar amounts in all segments. In contrast, [35 S]sulphate-labelled mature mucin appeared to display a sulphation gradient, increasing along the proximal-to-distal axis, while PAS staining showed equal amounts of mature RCM along this axis (Figures 10b and 10c). As the amounts of precursor and mature RCM are similar in all segments of the colon, we conclude that RCM is increasingly sulphated in the distal colon. (Figure 10 is representative of four independent experiments.) In mid-colon, a double band was detected by PAS staining, of which only the lower band was stained with [35 S]amino acids as well as [35 S]sulphate and corresponded in mobility to mature RCM. The upper band was not co-purified with RCM when RCM was isolated from mucosal scrapings from the whole colon by triple CsCl density gradient centrifugation, suggesting that it does not represent a mucin. The

identity of this upper PAS-stained band, which is apparently only present in the mid-colon, is currently under investigation.

DISCUSSION

Recent studies in our laboratory showed that the major mucin biosynthesized in normal human colon, designated HCM, is identical to MUC2 [23]. Because of the sequence homology of MUC2 with rat MLP [7,10,11] and with the rat MUC2 homologue [15], we studied RCM at the biochemical and cell biological level. Our goal was to characterize RCM and to reveal possible analogies with HCM at these levels. Therefore, the same isolation procedure was followed as previously used for HCM [23]. Great care was taken to prevent proteolytic breakdown of the less glycosylated protruding polypeptide parts [23]. The composition of RCM resembled other preparations of rat intestinal mucins [5,38]. Moreover, purified RCM appeared similar to purified HCM in buoyant density, chemical composition, molecular mass and mobility on SDS/PAGE.

With polyclonal anti-RCM, which was shown to react primarily with the protruding polypeptide parts of the mucin, it was possible to study the biosynthesis of RCM. RCM is synthesized as a precursor protein with an apparent molecular mass of about 600 kDa. This protein most likely represents an RCM precursor present in the RER, as it has the following properties: (1) it can be metabolically labelled with radiolabelled amino acids; (2) it contains high mannose *N*-glycans; (3) it cannot be stained by PAS; (4) it is not metabolically labelled with [³⁵S]sulphate or [³H]galactose; (5) it is not secreted into the medium; and (6) it is probably converted over time into a mature glycoprotein with an apparent molecular mass on SDS/PAGE of about 650 kDa. This '650 kDa', [³⁵S]amino acid-labelled glycoprotein most likely represents the mature mucin: (1) it can be stained by PAS; (2) it can be labelled metabolically with [³⁵S]sulphate or [³H]galactose; (3) it is secreted into the medium; and (4) it co-migrates with isolated RCM on SDS/PAGE.

The difference in mobility between the precursor and the mature RCM on SDS/PAGE is surprisingly small. Mucin is known to become heavily O-glycosylated and sulphated, which greatly enhances its molecular mass, far more than is apparent on SDS/PAGE. Interestingly, this small difference between precursor and mature mucin was also observed for HCM [23]. Most likely the mobility of RCM as well as HCM is anomalous, when compared with 'normal' proteins, due to the high carbohydrate content and to the highly negative charge of the mucins, caused by high amounts of sialic acid and sulphate. This may also explain why the absence of saturation with SDS has no influence on the mobility of RCM on PAGE.

As the molecular mass of the '650 kDa' band is probably anomalous, due to the high negative charge of the mature RCM, we sought to determine the molecular mass of the mature RCM independently. Gel filtration on Sepharose CL-2B of electroeluted [³H]galactose-labelled '650 kDa' band (reduced and carboxymethylated) also indicates that the mature mucin is in fact rather larger than 650 kDa, since spot blot analysis with anti-RCM reveals this material in the void volume, together with the [³H]galactose-labelled material (K. M. A. J. Tytgat, F. J. M. Opdam and J. Dekker, unpublished work). However, a more accurate size could not be determined, although it did show that these mucins are considerably larger than human and rat gastric mucin [23,36,37]. Furthermore, slight variations in the physical properties of the gels used to analyse the mucins have an effect on the mobility of the mature mucin, but not on the mobility of the RCM precursor, with reference to the molecular mass markers.

Analysis on non-reducing SDS/PAGE indicated that the 600 kDa RCM precursor oligomerized over time into very high-molecular mass complexes. These time-dependent shifts in mobility strongly suggest that oligomerization occurs before O-glycosylation and sulphation. Indeed, mature unreduced RCM, either labelled with [³⁵S]sulphate or detected by PAS staining, consisted of very large disulphide-linked complexes, as detected by both SDS/PAGE and gel filtration. These observations are very similar to the oligomerization of rat gastric mucin, as previously shown [27,36]. Therefore, we suggest that the RCM precursor oligomerizes in the RER, before transport to the Golgi complex [27]. Moreover, human MUC2 and rat MLP both contain cysteine-rich domains in their respective C-termini, which have been shown to contain high homology with domains in prepro-von Willebrand's factor, implicated in disulphide-linked oligomerization [10,11,39]. Also, the N-terminus of the rat MUC2 homologue was shown to contain cysteine-rich domains, implicated in oligomerization [15].

The RCM precursor showed heterogeneity among individual rats, with apparent molecular mass varying between about 550 and 650 kDa. In some rats, the RCM precursor is visible as a double band, most likely representing two independent gene products, as the bands were equally intense after pulse-labelling and remained equally intense during a 4 h chase-incubation. This probably reflects expression of two different alleles, as has previously been shown for rat gastric mucin [24]. Heterogeneity between individual rats is most likely of genetic origin, and may reflect differences in number of tandem repeats, as described for the human MUC1 gene [40]. It is of interest that mature RCM did not exhibit heterogeneity in apparent molecular mass, as PAS staining of RCM of all individual rats revealed a similar mobility on SDS/PAGE. However, different molecular masses of the mature RCM from the individual rats could exist, as these differences may remain undetected, due to the anomalous mobility of mature RCM on SDS/PAGE.

Regional differences in RCM biosynthesis in the colon were studied in order to detect possible differences in RCM processing. Biosynthesis of the RCM precursor appeared constant along the proximal-to-distal axis, with respect to both amount and molecular mass. However, a sulphation gradient of mature mucin exists, increasing from proximal to distal. This difference in sulphation may have implications for the local functions of RCM.

Initially, the number and the relative amounts of mucins in rat colon were unknown. Therefore, in our studies care was taken to isolate all expressed colonic mucins, according to a buoyant density of about 1.4 g/ml, and to elicit one polyclonal antiserum against these pooled colonic mucins. On PAS staining, isolated mucins became visible as a single high-molecular mass band. In metabolic labelling experiments, one mucin precursor with an apparent molecular mass of 600 kDa was detected, which was converted into a '650 kDa' glycoprotein band, which was secreted and which co-migrated with the PAS-stained mature mucin. Also in rat colonic homogenates, the '650 kDa' mucin was the only mucin visible. This suggests that RCM is a major secretory mucin in the rat colon. However, it cannot be excluded that other mucins are also present, either of a different buoyant density or less abundant.

This study shows that RCM is the rat analogue of human MUC2. Biochemically and cell biologically RCM and HCM are very similar: RCM and HCM have approximately the same apparent molecular mass, as precursor proteins as well as mature mucins, both mucins are located in colonic goblet cells, biosynthesis of these mucins proceeds very similarly and both mucins are secreted. Most remarkable are the cross-reactivities

of anti-RCM towards purified HCM and of anti-HCM towards purified RCM, which are completely abolished after proteinase K-digestion. This indicates that RCM and HCM share homologous peptide epitopes, which are most likely located at non-O-glycosylated, protruding polypeptide sequences. For rat MLP and human MUC2, homology in both the C- and N-terminus has been shown [8,10,12]. As HCM was previously identified as MUC2, the cross-reactivities of these anti-sera demonstrate that RCM is homologous to human MUC2 [23]. This homology was also independently indicated by the immunoprecipitation of RCM and HCM by the monoclonal antibody WE9, which was also found to recognize a proteinase-sensitive epitope of RCM as well as of HCM. Anti-MUC2 is also capable of immunoprecipitating RCM precursor. Cross-reactivity can be explained by the fact that the threonine-rich regions of MLP and the repeated amino acid sequence of human MUC2 contain numerous homologous sequences of 3–6 amino acids [7,8,10,11,13,16,39]. As anti-MUC2 is a polyclonal antiserum, it possibly recognizes these small homologous sequences of MLP, again independently suggesting that MLP is identical with RCM.

Thus, the results of this study suggest that RCM is identical with MLP and that RCM is the rat homologue of human MUC2. Therefore, we propose to designate RCM rat MUC2. As rat and human MUC2 most likely represent functionally analogous mucins, the rat provides an excellent animal model for the study of colonic mucins under experimental conditions.

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