C-terminal domain of rodent intestinal mucin Muc3 is proteolytically cleaved in the endoplasmic reticulum to generate extracellular and membrane components

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Although human MUC3 and rodent Muc3 are both membrane-associated intestinal mucins, the present study has explored the possibility that rodent Muc3 might exist in soluble as well as membrane forms. No evidence was obtained for the existence of soluble splice variants; however, experiments with heterologous cells transfected with cDNA encoding the 381-residue C-terminal domain of rodent Muc3 showed that a definitive proteolytic cleavage occurs during processing in the endoplasmic reticulum. The products consisted of a V5-tagged 30 kDa extracellular glycopeptide and a Myc-tagged 49 kDa membrane-associated glycopeptide. Throughout their cellular transport to the plasma membrane, the two fragments remained associated by non-covalent SDS-sensitive interactions. Site-specific mutagenesis pinpointed the need for glycine and serine residues in the cleavage sequence Leu-Ser-Lys-Gly-Ser-Ile-Val-Val, which is localized between the two epidermal-growth-factor-like motifs of the mucin. A similar cleavage sequence (Phe-Arg-Pro-Gly ↓ Ser-Val-Val-Val, where ↓ signifies the cleavage site) has been reported in human MUC1 and analogous sites are present in human MUC3, MUC12 and MUC17. Thus early proteolytic cleavage may be a conserved characteristic of many membrane-associated mucins, possibly as a prelude to later release of their large extracellular domains at cell surfaces.

Key words: cleavage, post-translational, splice variants, transfection.

INTRODUCTION

Rodent Muc3 shares certain structural features with at least six other membrane-associated mucins: MUC4 and its rodent orthologue Muc4, MUC3, MUC12, MUC13 and the recently described MUC17. The shared features include their large size (> 300 kDa), extensive central tandem repeats (TRs) with O-linked oligosaccharides and, in the C-terminal domain, a sperm protein, enterokinase and agrin ('SEA') module and two (or three in the case of MUC13) separated epidermal-growth-factor (EGF)-like motifs [1–6]. These are followed C-terminally by a transmembrane region and cytoplasmic tail. MUC1, the first described and now extensively studied membrane-associated mucin, differs from those above in not having the EGF motifs, but it does contain a glycosylated central TR domain, a transmembrane region and a cytoplasmic tail [7].

The functions of membrane-associated mucins, as judged primarily from studies of MUC1 and rat Muc4 [previously called ascites sialoglycoprotein (ASGP)], are diverse, ranging from physiological roles in cell growth, differentiation, uterine embryo implantation, epithelial protection and bacterial attachment to pathological roles in masking recognition sites for natural killer cells and promotion of malignant cell metastases [7–12]. Therefore knowledge of mucin structures is important to understand the basis for their functional versatility.

The term 'membrane-associated mucin' may be a partial misnomer, because serum dissemination of soluble forms of membrane mucins is common in human malignancies and certain inflammatory bowel diseases [13–16]. Soluble forms of MUC1, MUC4 and MUC3 are presumed to arise from alternative splicing and/or endoproteolytic cleavage within their C-terminal domains. Soluble products of two splice variants described to date for MUC1 include MUC1/SEC and MUC1/Y. MUC1/SEC, found in breast-cancer cells as well as in serum obtained from breast-cancer patients [13,14], results from an alternative splicing event occurring in the 3′ extremity of the TR array. The resulting isoform possesses neither a transmembrane sequence nor a cytoplasmic tail. The MUC1/Y splice variant is one in which the entire TR domain has been deleted. Its expression has been reported in various epithelial tumours, including breast and ovarian cancers [17,18], but it has not been detected in adjacent normal tissue. To date, 24 distinct transcripts have been identified for human MUC4 by reverse transcription (RT)-PCR procedures [19–21]. These transcripts arise from a complex alternative splicing mechanism, mainly within the 3′ terminal region, except for the variants MUC4/X and MUC4/Y, which arise by alternative splicing within the central TR domain. MUC4/X and MUC4/Y are expressed in cancer tissue, including tumours of lung and pancreas [20,21]. To date, however, none of the MUC4 transcripts has been confirmed at a protein level.

Abbreviations used: AP, adapter primer; ASGP, ascites sialoglycoprotein; BCIP, 5-bromo-4-chloroindol-3-yl phosphate; BHK, baby hamster kidney; Caco-2 cells, human colonic adenocarcinoma cells; CFTR, cystic fibrosis transmembrane conductance regulator; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; FBS, fetal bovine serum; NBT, Nitro Blue Tetrazolium; NP40, Nonidet P40; pSec, pSecTag2/HygroA; RT, reverse transcription; TR, tandem repeat.

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Unlike human MUC4, splice variants of rodent Muc4 have not been detected, and the full-length membrane form is the only form present in rodents, as judged by RT-PCR experiments [22]. Despite this, there is clear evidence that both soluble as well as membrane-bound forms of Muc4 exist in normal rodent airways, mammary gland and colon [12,22]. The soluble form is assumed to be due to proteolytic cleavage at an unidentified C-terminal site [12,22]. Proteolytic cleavage near the C-terminus was also detected in human MUC1 [23,24], allowing a soluble form of the mucin to be released from the membrane. Thus mechanisms involved in the production of soluble forms of membrane mucins include both splice variants and proteolytic cleavage.

MUC3 undergoes alternative splicing to encode a family of proteins that can be membrane-bound or (potentially) secreted [25,26]. Four distinct splice variants have been described, the most prevalent encoding the full-length membrane form and three others encoding soluble forms. The soluble MUC3 variants, like those of human MUC4, remain to be confirmed at the protein level. There is no information available pertaining to rodent Muc3 splice variation or proteolytic processing.

Using antibodies to synthetic peptides corresponding to sequences in the C-terminal region of rodent intestinal Muc3, we have demonstrated previously [27] that most of the mucin was membrane-associated, but some appeared as a soluble luminal product. There was a significant increase of Muc3 in the lumen of cystic fibrosis transmembrane conductance regulator (CFTR) knockout [UNC ('University of North Carolina')$^{CFTR\ (-/-)}$] mice. These observations prompted us to search for splice variants encoding soluble forms of Muc3 and for evidence during biosynthesis of a proteolytic cleavage event within the C-terminal region of Muc3. With C-terminal domain constructs of rat Muc3 and epitope tags, transient transfections have been carried out in COS-1 and other cells, and the expressed products analysed with epitope-specific antibodies. Our results suggest that rat Muc3 undergoes early processing steps that in some respects resemble those described for human MUC1 and/or rat Muc4. Our findings contribute to an understanding of the cellular physiology of Muc3 and its potential role in the pathogenesis of mucin abnormalities in disease.

MATERIALS AND METHODS

Materials

Cell-labelling mix (Pro-mix [^{35}S]) containing L-[^{35}S]methionine and L-[^{35}S]cysteine was purchased from Amersham Biosciences (Oakville, ON, Canada). Protein A–agarose, Protein G–Sepharose 4B Fast Flow and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, U.S.A.). Protease-inhibitor-cocktail tablets (Complete™ and Complete™ minus EDTA), RIPA buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P40 (NP40), 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors] kit, N-glycosidase F [peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase] and substrate Nitro Blue Tetrazolium (NBT)/5-bromo-4-chloroindol-3-yl phosphate (BCIP) were from Roche Molecular Biochemicals (Laval, QC, Canada). His·Bind® nickel resin and buffer kit were purchased from Novagen (Madison, WI, U.S.A.), and NP40 was from ICN Biomedicals (Aurora, OH, U.S.A). Dulbecco's modified Eagle's medium (DMEM) and DMEM lacking methionine and cysteine, OptiMEM® I (containing less serum), fetal bovine serum (FBS), penicillin, streptomycin and LIPOFECTAMINE™ were purchased from Life Technologies (Burlington, ON, Canada). Plasmid vector pSecTag2/HygroA (pSec) and TA cloning kit were obtained from Invitrogen (Groningen, The Netherlands). Immobilon-P membranes and Ultrafree filters were obtained from Millipore (Bedford, MA, U.S.A.). Trizol reagent, Superscript II RNase H-reverse transcriptase and adapter primer (AP) were purchased from Gibco BRL (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.).

Antibodies

Monoclonal anti-Myc and anti-V5 antibodies were obtained from Invitrogen. Alkaline phosphatase-conjugated goat anti-(mouse IgG), alkaline phosphatase-conjugated goat anti-(rabbit IgG) and alkaline phosphatase-conjugated donkey anti-(chicken IgG) antibodies were purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Polyclonal anti-6279 antibody, raised in rabbits to a peptide sequence within the C-terminus of rat Muc3, and anti-TR antibody, raised in chickens to three sequential consensus TRs of rodent Muc3, have been described previously [27].

Preparation of RNA and RT-PCR

Total RNA was prepared from ileal scrapings of two adult male rats (Wistar) or mice (Balb/c) using Trizol reagent (Gibco BRL). First-strand cDNA synthesis was catalysed by Superscript II RNase H-reverse transcriptase and primed with an AP to initiate cDNA synthesis at the polyadenylated region of mRNA. The RT reaction was carried out at 42 °C for 50 min, followed by inactivation of the enzyme at 70 °C for 15 min. The target cDNA was amplified by PCR using gene-specific primers chosen from the 3′ region of mouse Muc3 cDNA [28]. Primer mS1 (sense), 5′ATGAACGGAGGGTTCTGGACA-3′ (nt 1912–1932), corresponds to a region encoding the EGF1 motif. Primers mUTR (antisense), 5′-TACCCATTCTAGGTAACTGCA-3′ (nt 3021–3045), and mA1 (antisense), 5′-CAACGATGTCATGACTACCTG-3′ (nt 3179–3199), were designed to correspond to coding and non-coding regions to detect splice variants. Rat target cDNA was amplified with primers rS1 (rat counterpart of mS1) and AP.

PCR utilized *Taq* polymerase (Amersham Biosciences) and consisted of denaturation at 94 °C for 2 min (1 cycle), followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C (or 60 °C) for 1 min, extension at 72 °C for 5 min, with a final extension at 72 °C for 5 min. Gene-specific primers for mouse glyceraldehyde 3-phosphate dehydrogenase ('GAPDH'; ClonTech, Palo Alto, CA, U.S.A.) were used as positive controls in PCR reactions. PCR products were separated on 1% (w/v) agarose and stained with ethidium bromide.

C-terminal domain constructs of rodent Muc3 (p50, p20, pTR and p20t)

Constructs and primer usage are summarized in Table 1 and the expected products in Figure 1. A cDNA (p50) for the C-terminal 380 amino acids of rodent Muc3 [29] was inserted into the multiple cloning site of the expression vector pSec (Invitrogen). This vector contains the murine Ig κ chain leader sequence, a Myc epitope and polyhistidine sequence at the 3′-end. PCR reactions were carried out using the following primers: rS1 (sense), 5′-CGAAGCTTATGC**CTGAACGGAGGGTACTGG**-3′; rS2 (sense), 5′-CGAAGCTTAggtaagcctatccctaaccctctcctcggtctcgattctacg**TGCCTGAACGGAGGG TACTGG**-3′; rS3 (sense), 5′-CGAAGCTTAaccaccactcctgatgtgaccaccactcctgatgtgaccaccactcctgatgtg**TGCCTGAACGGAGGGTACTGG**-3′; and rA1

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Table 1 Fusion peptides expected for constructs of rodent Muc3 C-terminal domain

His, polyhistidine.

Construct	Primers	N-terminal tag	C-terminal tag	Total amino acids	N-glycans
p50	rS1, rA1	–	Myc, His	434	8
pTR	rS3, rA1	–	Myc, His	450	8
p20	rS2, rA1	V5	Myc, His	448	8
p20t	rS2, rA1	V5	–	171	3

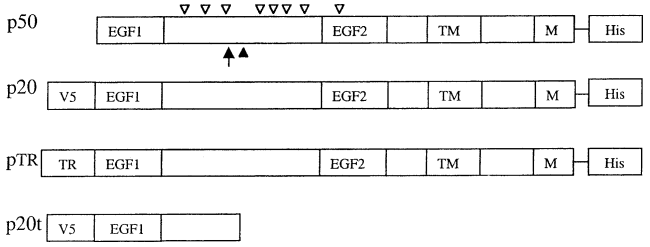

Figure 1 Schematic representation of rat Muc3 constructs

cDNA for the C-terminal 381 amino acids of rat Muc3 was cloned into the expression vector pSec. EGF1, EGF2 and TM represent two EGF-like domains and the transmembrane region respectively. M and His refer to Myc and polyhistidine epitope tags. Open arrowheads indicate the position of N-glycan consensus sites. Closed arrowhead indicates the approximate position of the Muc3 sequence designated '6279'. Black arrow indicates position of the sequence Leu-Ser-Lys-Gly-Ser-Ile-Val-Val. Constructs p20 and the truncated p20t contain a 5′ V5 epitope-tag sequence. pTR contains three sequential TRs encoding Thr-Thr-Thr-Pro-Asp-Val.

(antisense), 5′-CGCGGATCCGA**CAACGGTGTCATGGCC-AC**-3′. Sequences in bold represent rat Muc3 gene-specific sequences. For the three sense primers, these correspond to nt 1–15, and in primer rA1 nt 1121–1138. The bold sequence of rS1 was preceded by a *Hin*dIII site (CGAAGCTT) followed by TGC for cysteine. To maintain the correct reading frame, an extra nucleotide (A) was inserted between the *Hin*dIII site and TGC. Primers rS1, rS2 and rS3 are identical in their underlined regions. The 42 nt V5 epitope is shown in lower case in primer rS2. The 54 nt insertion (lower case) in primer rS3 represents three consensus TRs (Thr-Thr-Thr-Pro-Asp-Val) of rodent Muc3. The bold sequence of primer rA1 was preceded by a *Bam*H1 site (CGCGGATCC) and two extra nucleotides (GA) to maintain the Myc and polyhistidine sequences in the correct reading frame.

PCR was carried out using *Taq* polymerase and consisted of denaturation at 94 °C for 2 min (1 cycle), followed by 30 cycles at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 5 min, with final extension at 72 °C for 7 min. Following TA cloning, the PCR products were digested with *Hin*dIII and *Bam*H1, and ligated into the multiple cloning region of pSec. All inserts were confirmed by fluorescein-labelled DNA sequencing of both strands (Biotechnology Service Centre, The Hospital for Sick Children, Toronto, ON, Canada).

Mutations in p20 at the Gly-Ser-Ile-Val-Val site

Site-specific mutations were carried out on p20 using the Quickchange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) to modify the Leu-Ser-Lys-Gly-Ser-Ile-Val-Val motif present within the rodent Muc3 sequence (residues 122–129; Figure 1) [29]. The construct encoding mutant Leu-Ser-Lys-Ala-Ser-Ile-Val-Val was designated p20g/a, and that encoding mutant Leu-Ser-Lys-Gly-Ala-Ile-Val-Val was designated p20s/a. The primer pair used to produce p20g/a was 5′-CTGAGCAAAG**C**CAGTA TCGTGGTGG-3′ (sense) and 5′-CCACCACGATACTG**G**CTTTGCTCAG-3′ (antisense). The primer pair used to produce p20s/a was 5′-CTGAGCAAAGGC**GCC**ATCG TGGTGGAT-3′ (sense) and 5′-ATCCACCACGAT**GGC**GCCTTTGCTCAG-3′ (antisense). Nucleotides in bold represent the base-pair change that produced the required mutation. PCR reactions were carried out according to the kit protocol and mutations confirmed by DNA sequencing.

Cell culture and DNA transfection

COS-1 and human colonic adenocarcinoma (Caco-2) cell lines were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. Cells were cultured in DMEM supplemented with 10 % (v/v) FBS. Baby hamster kidney (BHK) cells (from American Type Culture Collection) were maintained in DMEM/Ham's F-12 medium supplemented with 10 % (v/v) FBS. Culture media also contained penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 and subcultured at a dilution of 1:10. At approx. 80–100 % confluence, cells were seeded into 3.5-cm-diameter tissue-culture dishes at a density of approx. 8×10^6 cells/dish. At 40–50 % confluence in the case of COS-1 or 50–60 % confluence in the case of Caco-2 and BHK cells, DNA transfections were carried out with 2 μg of plasmid and 10 μl of LIPOFECTAMINE™ in the presence of OptiMEM® I. Vector alone transfections served as a control in each experiment. Cells were incubated for 5 h with the transfection mixture, the mixture was replaced with fresh medium and the incubation continued for a total of 40 h, with one change of medium at 16 h.

SDS/PAGE and Western-blot analysis

Cell media (2 ml) were collected from each dish, mixed with complete protease-inhibitor cocktail, centrifuged at 500 ***g*** for 5 min at 4 °C, and the supernatant was concentrated by filtration to 10 % of its original volume. Cells were washed with PBS and lysed in 200 μl of SDS-sample buffer. Cell lysates or media were subjected to SDS/PAGE (4–20 % gradient polyacrylamide gels; Novex, San Diego, CA, U.S.A.) under reducing and non-reducing conditions (i.e boiling for 3 min with or without DTT), then transferred on to Immobilon-P membranes. Membranes were blocked for 1 h at 22 °C with 20 mM Tris/HCl buffer (pH 7.4) containing 0.15 M NaCl and 3 % (w/v) BSA. Immunostaining was carried out at 4 °C overnight with anti-Myc or anti-V5 antibodies, followed by a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-(mouse IgG) antibody for 1 h at 22 °C. In some experiments, rabbit polyclonal anti-6279 antibody was used as the primary antibody and detection was carried out with the alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibody. Positive bands were detected with the NBT/BCIP substrate system. Seeblue™ plus 2 (Invitrogen) was used as molecular-mass standards.

Metabolic labelling and immunoprecipitation of expressed proteins

At the end of the transfection period (40 h), COS-1 or Caco-2 cells were pulse-labelled (15 min) with [^{35}S]-Pro-mix and chased

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in unlabelled media for either 0 or 2 h as described previously [30], except that the chase medium contained 0.24 mg/ml cysteine and 0.15 mg/ml methionine. Cells were lysed in 300 μl of RIPA buffer, centrifuged and supernatants precleared with 50 μl of Protein A–agarose. Specific protein products were immunoprecipitated during a 16 h incubation at 4 °C with anti-V5 or anti-Myc antibodies in the presence of 50 μl of Protein A–agarose (for anti-V5 antibody) or Protein G–Sepharose (for anti-Myc antibody). Immunoprecipitates were washed twice in RIPA buffer and once in 10 mM Tris/HCl buffer (pH 7.4) containing 0.1 % NP40, and then subjected to SDS/PAGE. Gels were rinsed with distilled water, fixed in 30 % methanol/10 % acetic acid, dried and exposed to X-ray film (Kodak X-Omat XAR-5) for 1–3 days.

Affinity purification of His-tagged proteins

His-tagged proteins in cell lysates were affinity purified using the His·Bind® nickel resin according to the supplier's instructions. Briefly, cells were lysed by homogenizing in binding buffer [20 mM Tris/HCl buffer (pH 7.9) containing 0.5 M NaCl, 5 mM imidazole and 0.5 % NP40]. Following centrifugation at 1000 ***g*** at 22 °C for 5 min, the supernatant was incubated with the resin at 4 °C for 1 h with gentle rotation. The resin was washed thoroughly with a solution containing 60 mM imidazole, 0.5 M NaCl and 20 mM Tris/HCl (pH 7.9) to remove loosely or non-bound proteins. His-tagged protein products were eluted with the same buffer adjusted to contain 1 M imidazole, and subjected to SDS/PAGE and Western blotting. In some cases, deglycosylation was performed prior to SDS/PAGE.

Deglycosylation of His-tagged proteins with N-glycosidase F

Samples were boiled for 5 min in 1 % (w/v) SDS, incubated at 37 °C for 16 h in the absence or presence of 20 units of N-glycosidase F in 10 mM sodium phosphate buffer (pH 7.0) containing 30 mM EDTA and 0.7 % NP40. Samples were filter-concentrated and subjected to SDS/PAGE followed by Western blotting and immunodetection.

Detection of rodent Muc3 cleavage products in brush-border membranes

Rat intestinal brush-border membranes were prepared as described previously [27], and subjected to SDS/PAGE and Western blotting using anti-6279 or anti-(TR) antibodies. Positive bands were detected with second antibody conjugates and the NBT/BCIP substrate system.

RESULTS

Splice variants of rodent Muc3 were not detected

RT-PCR experiments were conducted using ileal RNA of mice and primer pairs from the translated as well as untranslated region of mouse Muc3 cDNA (Figure 2). The RT-PCR product of each primer pair was a single band, having the same size as expected if no splice variants existed, namely 1140 bp for primer pair mS1 and mA1, 1285 bp for primer pair mS1 and mUTR and 1690 bp for primer pair mS1 and AP (Figure 2). Thus there was no evidence to support the suggestion that splice variation(s) accounts for a potential soluble form of normal rodent Muc3. Since rat and mouse Muc3 cDNA share over 85 % identity in their 3′ region [28,29], the absence of splice variants is presumed to apply to both of the rodent *Muc3* genes. This was supported by RT-PCR experiments in which a single amplimer was obtained from rat ileal RNA using primers rS1 and AP.

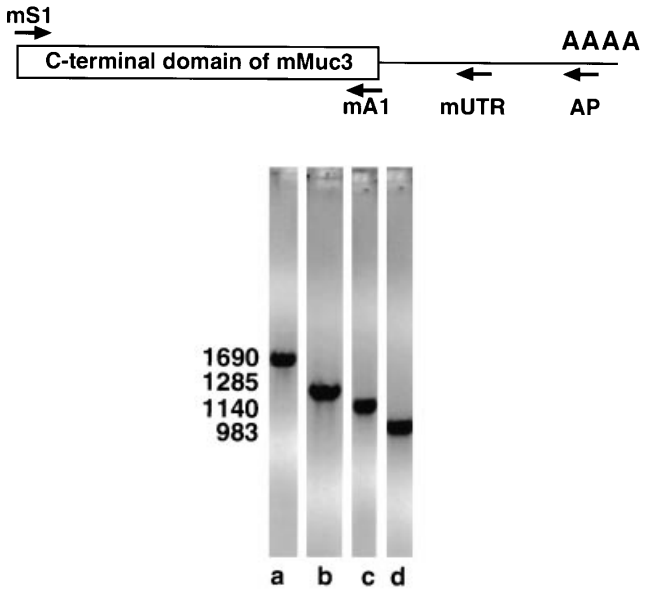

Figure 2 RT-PCR products of mouse small intestinal RNA

RT-PCR products from primer pairs mS1 and AP (lane a), mS1 and mUTR (lane b) and mS1 and mA1 (lane c) were separated on 1 % (w/v) agarose gels and stained with ethidium bromide. Lane d represents the product of a glyceraldehyde-3-phosphate dehydrogenase primer pair as a control. Numbers represent the size (bp) of the PCR products with reference to *Hin*dIII-digested λDNA fragments as standards.

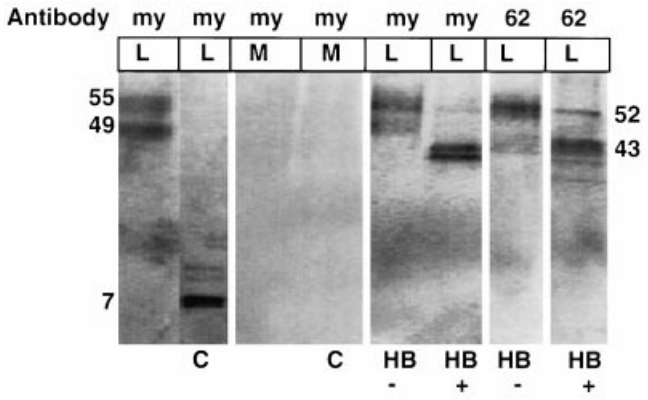

Figure 3 Expression of p50 in COS-1 cells

Products of transfected cells in cell lysates (L) or media (M) were detected by SDS/PAGE and Western blotting with anti-Myc (my) or anti-6279 (62) antibodies. C, pSec vector controls. HB represents lysates affinity purified on a His·Bind® nickel resin, followed by the absence (−) or presence (+) of digestion with N-glycosidase F prior to SDS/PAGE. Numbers represent molecular mass (kDa).

Subsequent experiments explored the possibility that post-translational proteolytic processing in the C-terminal domain of Muc3 might lead to a soluble form of intestinal Muc3.

Transfection of COS-1 cells with construct p50 yields two products

Construct p50, which encodes the entire C-terminal domain of rat Muc3 (Figure 1), was expressed in COS-1 cells, and lysates and media were subjected to SDS/PAGE and Western blotting

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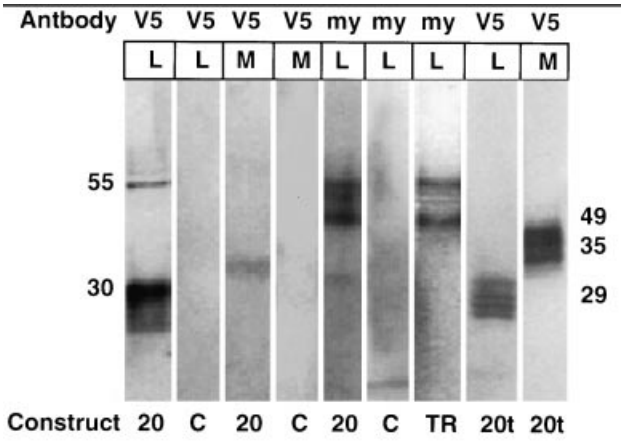

Figure 4 Expression of p20, pTR and p20t in COS-1 cells

Cell lysates (L) or spent media (M) of constructs p20 (20)-, pTR (TR)- and p20t (20t)-transfected cells were detected by SDS/PAGE and Western blotting with anti-V5 (V5) or anti-Myc (my) antibodies. C, pSec vector controls. Numbers indicate molecular mass (kDa).

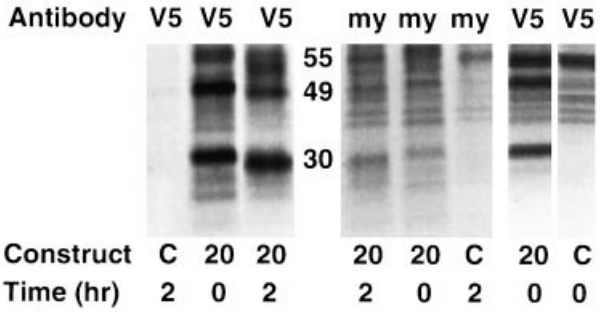

Figure 5 Immunoprecipitated products of p20 in pulse–chase experiments with COS-1 and Caco-2 cells

COS-1 cells transfected with construct p20 (20) were pulsed for 15 min with Pro-mix [^{35}S] and chased for either 0 or 2 h. Cell lysates were immunoprecipitated with anti-V5 (V5) or anti-Myc (my) antibodies, separated by SDS/PAGE and detected by autoradiography. Caco-2 cell products of p20 expression are shown in the two right-hand lanes. C indicates pSec vector controls. Numbers indicate molecular mass (kDa).

using the anti-Myc antibody. As shown in Figure 3, two broad immunoreactive bands appeared at approx. 55 and 49 kDa in cell lysates (lanes labelled L in Figure 3). The pSec vector alone and non-transfected COS-1 cells gave no immunoreactive bands in this region (results not shown). The 7 kDa band seen in the pSec vector control (lane labelled C in Figure 3) corresponded to the Myc epitope. The media of p50-transfected cells were negative, suggesting no secretion or release of Myc-labelled components. Reactivity with the antibody against the rodent Muc3 peptide, anti-6279, showed the 55 kDa band and a less-intense band between 43 and 45 kDa (see later).

Both 55 and 49 kDa products contain N-linked oligosaccharides

The two bands at 55 and 49 kDa were presumed initially to represent two different glycoforms of the same rodent Muc3 polypeptide product. To test this hypothesis, cell lysates were first purified by the His·Bind® nickel resin, then incubated with N-glycosidase F for 16 h and subjected to SDS/PAGE and Western blotting (Figure 3, lanes HB). Following deglycosylation, the major anti-Myc-reactive bands were reduced in size to 41–43 kDa. The original 55 kDa band became much less intense, but a single band at 52 kDa was detected by using the anti-Myc antibody. The anti-6279 antibody readily recognized the deglycosylated 52 kDa band and a band at 43 kDa. Two interpretations were considered: that either the two products at 55 and 49 kDa were glycoforms, but deglycosylation was incomplete for the 55 kDa band, or the original products at 55 and 49 kDa were not just glycoforms, but represented different glycosylated peptide cores, each containing the 6279 peptide epitope and the same Myc- and polyhistidine-positive C-terminal epitope tags. Since construct p50 lacked an N-terminal epitope tag, further experiments were required to determine if the 55 and 49 kDa glycopeptides differed with respect to their N-terminal sequence.

Evidence for cleavage of the rodent Muc3 polypeptide core

As shown in Figure 1, construct p20 is flanked on either side by epitope tags (V5 and Myc). COS-1 cells were transfected with p20, and cell lysates and medium were subjected to SDS/PAGE and Western blotting using anti-V5 and anti-Myc antibodies (Figure 4). The anti-V5 antibody detected products in cell lysates at 55 and 30 kDa (with a smear below), the dominant product being the 30 kDa band. A minor amount of the 30 kDa product was released into the cell medium and was detected at approx. 32 kDa. Western blots using the anti-Myc antibody recognized the 55 and 49 kDa bands, but not the 30 kDa band. It was concluded, therefore, that the 55 kDa band represents the full-length C-terminal peptide of rodent Muc3. Cleavage of this domain produced the C-terminal anti-Myc-positive fragment of 49 kDa and the N-terminal V5-positive fragment of 30 kDa. The anti-6279-positive products seen earlier (Figure 3) at approx. 52 and 43 kDa (but not 30 kDa) were presumed to represent the most sparsely glycosylated glycoforms of the 55 and 49 kDa products. Recognition of these products by the antibody suggests that the cleavage occurred within or N-terminal to the 6279 epitope (arrowhead in Figure 1).

Presence of TRs did not prevent cleavage

COS-1 cells were transfected with construct pTR to determine if the presence of TRs at the N-terminus of the rodent Muc3 domain would prevent peptide cleavage. SDS/PAGE and Western blots of cell lysates using anti-Myc antibody, however, showed the same two immunoreactive bands (55 and 49 kDa; Figure 4). Thus TRs did not confer a protective effect, unlike the TR-mediated anti-proteolytic effect described by Loomes et al. [31] for a MUC6-containing hybrid protein.

Cleavage of rodent Muc3 occurs during an early stage of biosynthesis

COS-1 cells were transfected with p20, metabolically labelled with Pro-mix [^{35}S] for 15 min and chased in non-radioactive media for either 0 or 2 h. Cell lysates and media were immunoprecipitated with anti-V5 and anti-Myc antibodies, and the products were separated on SDS/PAGE (Figure 5). Even at time 0 of the chase, three major products were noted (midpoints at 57, 50 and 32 kDa) in cell lysates, seen best using the stronger anti-V5 antibody, but also detected with anti-Myc antibody. After a 2 h unlabelled chase, the bands increased slightly in mobility (to 55, 49 and 30 kDa respectively), possibly reflecting further biosynthetic processing. Thus cleavage of the C-terminal domain of rodent Muc3 occurs within 15 min of protein synthesis, presumably in the endoplasmic reticulum. Since both antibodies

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precipitated all three products, the two cleavage products must have remained associated until they were separated by SDS/PAGE. Faint bands below the 30 kDa and 49 kDa positions (observed using both antibodies) may be non-specific bands, degradation products or lower glycoforms, but were not investigated further. Immunoprecipitation of cell media yielded no bands at either time period (results not shown).

Cleavage of Muc3 domain is not restricted to COS-1 cells

To demonstrate that cleavage of rodent Muc3 was not specific to host COS-1 cells, p20 transfections were also carried out in Caco-2 cells, a cell line that is known to produce human mucin MUC3 [32]. After a pulse period of 15 min with Pro-mix [^{35}S], immunoprecipitation with the anti-V5 antibody showed immunospecific bands at 49 and 30 kDa (Figure 5, two right-hand lanes labelled V5). The specifity of the 55 kDa band observed in these experiments was dubious, because a band in this position was also seen in vector-only control samples (lanes labelled C in Figure 5). The 55 kDa band was not seen in Western blots of control or test samples of Caco-2 lysates assayed at 24 h post-transfection. Thus we do not have a satisfactory explanation for its appearance in immunoprecipitates. Despite this, the results confirmed, however, that rodent Muc3 yields the same 49 and 30 kDa cleavage products in Caco-2 cells as in COS-1 cells. Similar cleavage products were also detected by Western blots of p20 transfectants of BHK cells (results not shown).

Two fragments of rodent Muc3 remain associated following cleavage

It was expected that the 30 kDa N-terminal cleavage product would be soluble (non-membrane bound) and released into the medium of transfected COS-1 cells. However, inability to detect more than trace amounts of this product in the media by 2 h of pulse–chase or by Western blotting, even at 24 h post-transfection, suggested that the 30 kDa fragment remained associated with the membrane-attached 49 kDa fragment within the cells even after cleavage of the full-length peptide. Two different approaches were taken to confirm this interpretation: (1) expression of a designed C-terminally truncated product with no attachment to other products; and (2) use of a His·Bind® nickel resin to determine if it would capture both fragments in p20 transfectants.

Transfection of COS-1 cells with p20t

The C-terminally truncated construct, p20t (Figure 1), lacking both the transmembrane and cytoplasmic tail of Muc3, was expected to express a product of approximately the same size as the 30 kDa fragment of p20. This was confirmed by showing that a V5-immunoreactive band appeared (Figure 4, lanes labelled 20t) as a broad band of 25–32 kDa (midpoint 29 kDa). An intense band was also observed in the media (midpoint 35 kDa), suggesting that the cell product had been further glycosylated and actively secreted. Therefore, under normal conditions when the full-length p20 product is cleaved, the N-terminal 30 kDa fragment is not secreted because it still remains associated with the C-terminal (49 kDa) membrane-tethered fragment. Without this attachment the extracellular domain would be secreted, as was the case for the product of p20t.

Affinity purification of fragments by using a His·Bind® nickel resin

If the 49 kDa Myc-positive and 30 kDa V5-positive fragments of the rodent Muc3 domain did not remain associated after cleavage, application of p20 cell lysates to a His · Bind® nickel resin should yield only the polyhistidine-bearing 49 kDa C-terminal fragment and any remaining non-cleaved 55 kDa product. If they remained associated, however, both cleavage products should appear on subsequent SDS/polyacrylamide gels. As shown in Figure 6, the latter was observed. Anti-V5 antibody recognized bands at 55 and 30 kDa (Figure 6, lane a), whereas anti-Myc recognized bands 55 and 49 kDa (Figure 6, lane b). Thus the cleavage fragments remained associated during co-purification through the His·Bind® nickel resin.

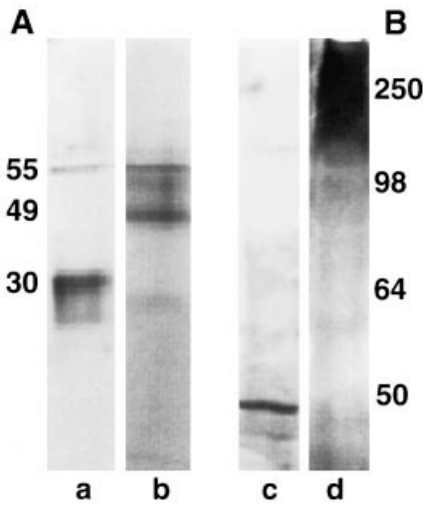

Figure 6 Muc3 cleavage fragments present in COS-1 cell transfectants and rat intestinal brush-border membranes

(**A**) Cell lysates of p20-transfected COS-1 cells were affinity purified on a His·Bind® nickel resin and subjected to SDS/PAGE (4–20% gels) and Western blotting using anti-V5 (lane a) and anti-Myc (lane b) antibodies. (**B**) Rat intestinal brush-border membranes were subjected to SDS/PAGE (8% gels) and Western blotting using anti-6279 (lane c) and anti-(TR) (lane d) antibodies. Numbers represent molecular mass (kDa).

Identification of the site of cleavage in the rodent Muc3 C-terminal domain

As stated above, the pattern of anti-6269 reactivity suggested that cleavage may have occurred within or N-terminally adjacent to the 6279 epitope site (arrowhead in Figure 1). This is consistent with an N-terminal fragment of approx. 30 kDa and with the observed size of product from the construct p20t. Analysis of the sequence between the two EGF motifs of rodent Muc3 and human MUC3 revealed only one five amino acid motif, Gly-Ser-Ile-Val-Val, that was conserved between the two species. This motif is in a position that, if cleaved, would release an N-terminal fragment of approx. 30 kDa (Figure 1, arrow). A similar proteolytic site (Phe-Arg-Pro-Gly↓Ser-Val-Val-Val, where ↓ signifies the cleavage site) has recently been identified in human MUC1 [24]. These considerations prompted us to introduce mutations at the glycine and serine residues within the Gly-Ser-Ile-Val-Val motif encoded by the p20 construct. Figure 7 shows that mutation of glycine to alanine partially prevented cleavage (Figure 7, lane e versus the wild-type in lane f), and mutation of serine to alanine totally prevented the appearance of the 30 kDa cleavage product (Figure 7, lane d versus lane f). Thus it is highly likely that proteolysis of the rodent Muc3 domain occurs at the Gly–Ser site of the Gly-Ser-Ile-Val-Val motif.

The non-cleaved high-molecular-mass Muc3 products from p20g/a and p20s/a transfections were not only intense, but they

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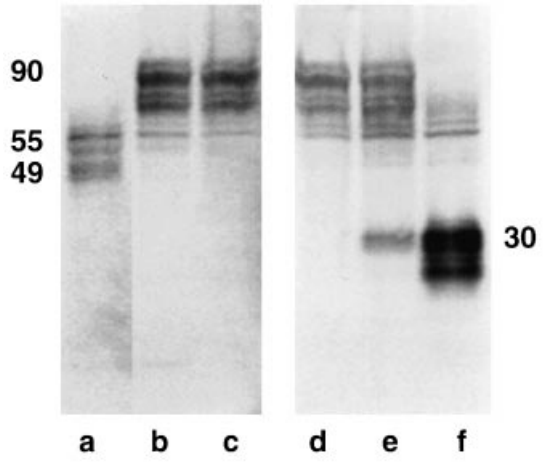

Figure 7 Expression of p20, p20g/a and p20s/a in COS-1 cells

Lysates of cells transfected with p20 (lanes a and f), p20g/a (lanes b and e) and p20s/a (lanes c and d) were subjected to SDS/PAGE and Western blotting using anti-Myc (lanes a–c) and anti-V5 (lanes d–f) antibodies. Numbers indicate molecular mass (kDa).

appeared as broad bands or a ladder of bands ranging in molecular mass from 55 to 90 kDa (Figure 7, lanes b–e). All were recognized by anti-V5 and anti-Myc antibodies, thus represent full-length glycoforms. Recent preliminary experiments in which these samples have been digested with N-glycanase confirm this interpretation (R. Wang, I. A. Khatri and J. F. Forstner, unpublished work).

In vivo validation of the presence of C-terminal cleavage products of Muc3 in brush-border membranes

Brush-border membranes of rat intestine were subjected to SDS/PAGE and Western blotting using anti-6279 and anti-(TR) antibodies (Figure 6B). A band at 48 kDa was detected with the anti-6279 antibody (Figure 6, lane c), and a high-molecular-mass glycoprotein was detected with the anti-(TR) antibody (lane d). These results indicate that, as with the p20 product, full-length Muc3 mucin *in vivo* is also cleaved within the C-terminal region, but the large extracellular domain of the mucin remains associated with the 48 kDa membrane-embedded fragment in the apical cell membrane. SDS breaks the interaction, allowing the products to separate during electrophoresis.

DISCUSSION

Variants of human MUC1, MUC3 and MUC4 arise from alternative splicing mechanisms, mainly within the unique 3′ terminal (non-TR) region of each gene, giving rise to membrane-associated forms and larger highly glycosylated soluble forms. Although rodent counterparts of these three mucins are very similar in their C-terminal regions, 3′ splice variants of rodent Muc1 and Muc4 have not been detected and, in the present study, none were detected for rodent Muc3. It would appear, therefore, that rodents have simpler or fewer regulatory controls on transcription of membrane mucin genes, thus accounting for the absence of multiple alleles.

Results of expression of the C-terminal domain of rodent Muc3 in heterologous cells leave little doubt that a proteolytic cleavage occurs during biosynthesis at a position located between the two EGF sites. The resultant fragments, however, remained associated even during cell lysis in solutions containing 0.1 % SDS, immunoprecipitation by antibodies directed to only one of the two fragments or capture by a nickel resin specific for the polyhistidine tag on the C-terminal fragment. Dissociation required SDS/PAGE in the presence of 2 % (w/v) SDS and was not influenced by the absence or presence of reducing agents. Thus the association of the two fragments is probably through non-covalent interactions. These interactions persist throughout cellular processing and transport, since the two fragments were found within cells at 0 and 2 h of chase in pulse–chase experiments, and only trace amounts of the extracellular domain fragment (30 kDa) was noted occasionally in the medium of transfected cells. The same cleavage and persistent association of cleavage fragments probably occurs in full-length Muc3 *in vivo*, since purified brush-border membranes of rat intestine exhibited both extracellular and membrane-associated fragments.

Cleavage of the C-terminal domain of rodent Muc3 appears to follow a course similar to proteolytic separation of extracellular and membrane subunits described for human MUC1 and rat Muc4 (ASGP) during their processing in the endoplasmic reticulum [33,34]. In both cases, the extracellular and membrane subunits remain associated by non-covalent interactions that persist after cleavage and throughout intracellular processing and transport to the cell surface. Both MUC1 and rat Muc4 reach the apical surface in an incompletely glycosylated state, and additional oligosaccharides are added to the mucins in subsequent waves of endocytosis and recycling through the *trans*-Golgi network [35,36]. Recycling has not been tested in Muc3, but might be expected in the light of its similarity to the other membrane mucins and multiple glycoforms. The significance of recycling is not well understood, but it has been proposed that differentially glycosylated forms of membrane-associated mucins may have distinct roles in processes such as cell–cell adhesion, metastatic potential of tumours and recognition phenomena [36]. The possibility of recycling of rodent Muc3 is therefore an interesting topic for future research.

The proteolytic site differs in MUC1 and rat Muc4. Muc4 is cleaved at an Asp–Pro site near the junction of the two subunits ASGP1 (the large extracellular domain) and ASGP2 (the C-terminal subunit containing the transmembrane and cytoplasmic tail regions) [2,34]. MUC1 is cleaved at a Phe-Arg-Pro-Gly ↓ Ser-Val-Val-Val motif (where ↓ signifies the cleavage site) present in its C-terminal region 60–67 residues upstream of the transmembrane region [24]. The protease responsible for MUC1 cleavage has not been identified, but is thought to be a kallikrein-like serine protease [37,38]. Rat Muc3 lacks the Asp–Pro site found in Muc4, but has a MUC1-like sequence, Leu-Ser-Lys-Gly-Ser-Ile-Val-Val, located 120–127 residues upstream of the transmembrane region. As shown in the present study, cleavage gave rise to an N-terminal fragment having a size expected from a cleavage reaction near or at this site (30 kDa), and site-specific mutations at glycine and serine residues in this motif prevented cleavage. It is therefore highly likely that rodent Muc3 is cleaved at the Leu-Ser-Lys-Gly ↓ Ser-Ile-Val-Val (where ↓ signifies the cleavage site) site during biosynthesis.

In the absence of cleavage (with constructs p20g/a and p20s/a), the rodent Muc3 domain appeared as a broad band (ladder) ranging from 55–90 kDa. Since the entire ladder was recognized by antibodies specific for both N- and C-terminal epitopes (V5 and Myc), and since there are eight potential N-glycans, the ladder represents different glycoforms. With cleavage, as in the p50, p20 or pTR construct products, there was usually a small amount of residual uncleaved 55 kDa product, which was never as prominent as the cleavage fragments. The fully deglycosylated

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product is approx. 52 kDa, judging from the product size after N-glycosidase F treatment, and the 55 kDa component represents a minimally glycosylated immature form. Proteolytic cleavage is presumed to occur as further glycosylation proceeds.

The stable association of the cleavage products of the rodent Muc3 domain and the almost total lack of secretion of the extracellular fragment (30 kDa) into the media, suggest that there may be a further reaction needed for release of the soluble extracellular domain of Muc3. There appears to be no failure of the cell secretory mechanism itself, since the product of the truncated construct p20t was secreted by transfected COS-1 cells. In the case of MUC1 and rat Muc4, it has been proposed that an additional unidentified step(s) (beyond the initial cleavage to produce membrane and extracellular domain subunits) is also needed to release a soluble form of MUC1 or Muc4 from the cell surface [22,33]. This may or may not involve a second proteolytic step. For example, both membrane-associated and soluble forms of Muc4 have been found along the apical lining of tracheal cells. The soluble form remains loosely adsorbed on the cell surface, but can be removed by tracheal rinsing [12]. Whether this is also true of intestinal Muc3 *in vivo* is not known. Thus the mechanism(s) responsible for an increase of Muc3 in the intestinal lumen of CFTR knockout mice [27] is as yet unexplained. However, initial proteolytic generation of two fragments representing the extracellular domain and the transmembrane–cytoplasmic tail domain is probably an important prerequisite for later release of soluble Muc3 at the luminal surface. The nature of the non-covalent association linking these fragments and the possible involvement of an additional intracellular or extracellular protease will be investigated in future studies.

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