SEA (sea-urchin sperm protein, enterokinase and agrin)-module cleavage, association of fragments and membrane targeting of rat intestinal mucin Muc3

Ismat A. KHATRI*, Rongquan WANG† and Janet F. FORSTNER*[1]

*Research Institute, Department of Structural Biology and Biochemistry, The Hospital for Sick Children, and Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada, and †Department of Gastroenterology, Southwestern Hospital, Third Military Medical University, Chongqing, Peoples' Republic of China

In a previous study we showed, by transient expression studies in COS-1 cells, that the C-terminal domain of rat intestinal membrane mucin Muc3 was cleaved between glycine and serine within a GSIVV (one-letter) amino acid sequence during its residence in the endoplasmic reticulum. The extracellular domain fragment remained linked to the membrane-associated fragment by non-covalent interactions. The present study demonstrates that cleavage depends not only on the presence of the G/SIVV site (where G/S is the glycine↓serine cleavage site), but also on more distant C-terminal sequences in the SEA (sea-urchin sperm protein, enterokinase and agrin) module. Inhibition of N-glycosylation by tunicamycin treatment of transfected cells did not prevent re-association of fragments, although cleavage was partially impaired, as some of the non-glycosylated, non-cleaved products were seen to accumulate in cells. Membrane targeting of the Muc3 domain and its cleavage products occurred in transfected cells and was not impaired in mutants in which the cleavage site was mutated. Targeting was also not impaired for products devoid of N-linked oligosaccharides. Our studies thus indicate that (a) cleavage within the SEA module of rat Muc3 requires participation of peptide sequences located C-terminal of and distant from the cleavage site, (b) re-association of the fragments requires the SEA module, but is independent of N-linked oligosaccharides, and (c) membrane targeting of the mucin is independent of the SEA-module-cleavage reaction.

Key words: expression, immunolocalization, membrane mucin, peptide cleavage, SEA (sea-urchin sperm protein, enterokinase and agrin) module.

INTRODUCTION

Membrane-tethered mucins and their soluble counterparts are found in epithelial linings throughout the body. On the basis of knowledge of their structure, surface location and expression patterns, it is believed that these molecules play an important role in barrier function. In addition to presenting extended, heavily glycosylated domains on the cell surface, structural features within the C-terminal regions suggest that many if not all membrane mucins may have the ability to shed their extracellular domain and/or to engage in cell-signalling processes via their cytoplasmic tail [1,2]. Human mucins MUC1 and MUC4, for example, have been implicated in the regulation of cell adhesion, growth and differentiation [3,4]. Excessive shedding or release of extracellular domains of mucins is also a prominent characteristic of metastatic carcinoma, inflammatory bowel disease and cystic fibrosis [5–9].

In a recent paper [10] we presented evidence that the expressed C-terminal domain of the rodent membrane mucin Muc3 (construct p20) undergoes proteolytic cleavage during an early period of biosynthesis in the endoplasmic reticulum (ER). The N-terminal cleavage fragment of 30 kDa was not secreted, but remained associated with the 49 kDa C-terminal membrane-tethered fragment by non-covalent disulphide-bond-independent interactions. The same process apparently occurs *in vivo*, since the corresponding two fragments of rodent mucin Muc3 were detected in isolated brush-border membranes. The biological purpose of the cleavage and re-association of fragments is not understood, but is possibly important for later release of the soluble extracellular domain into the intestinal lumen. Alternatively, cleavage may represent a mechanism by which the extracellular domain is primed for its later role as a ligand for a cell-surface receptor. These speculations are based on demonstrations by others [1,4,11,12] that human MUC1 and rat Muc4 can be solubilized by a proteolytic cleavage near the C-terminus, and can interact with receptors on epithelial cells.

Using a series of mutant constructs, we established that the site of cleavage in rat intestinal mucin Muc3 (rMuc3) was in a sequence GSIVV located between the two epidermal-growth-factor (EGF)-like motifs in a region now called the 'SEA (sea-urchin sperm protein, enterokinase and agrin) module' [13]. SEA modules are defined as conserved 80–110-amino-acid motifs that occur in extracellular matrix or membrane-associated proteins having diverse functions, but all characterized by being heavily O-glycosylated. In many G-protein-coupled receptors, SEA modules are found between EGF motifs with conservation of the GSVVV (amino acid one-letter code) proteolytic cleavage site [14]. Membrane mucins such as MUC1, MUC3, MUC12, MUC13 and MUC17, have also been shown to contain a SEA module motif in their carboxyl terminal domain, and to have the consensus cleavage site GSVVV [15,16]. Experimental proof of cleavage at this site, however, is so far restricted to rMuc3 [10], MUC1 [17] and Ig-hepta, a non-mucin plasma-membrane receptor [18]. In all cases, the cleaved fragments re-associate and the extracellular

Abbreviations used: G/S, glycine↓serine cleavage site in one-letter amino acid code; rMuc3, rat intestinal mucin Muc3; N-glycosidase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase; EGF, epidermal growth factor; ER, endoplasmic reticulum; SEA, sea-urchin sperm protein, enterokinase and agrin; TM, transmembrane region.

[1] To whom correspondence should be sent, at The Hospital for Sick Children, 555 University Avenue, Toronto, ON, Canada M5G 1X8 (e-mail jfforst@sickkids.on.ca).

domain thus remains attached (indirectly) to the membrane. It has been proposed by Bork and Patthy [13] that SEA modules have an important adhesive function, possibly by facilitating binding to carbohydrate residues on neighbouring glycoproteins or proteoglycans. Wreschner et al. [15] have proposed that SEA modules (by undergoing cleavage) permit a protein to function as both a ligand and a receptor at the cell membrane (in a partnership) to elicit a signalling cascade.

In a previous study, a C-terminally truncated construct of rMuc3, designated p20t, which lacks the transmembrane region and cytoplasmic tail, was expressed in COS cells. The product was rapidly secreted into the medium, appearing as a broad band with a mid-point molecular mass of 35 kDa [10]. Although p20t contained the GSIVV site and 22 amino acids C-terminal to this site, it was not certain that G/SIVV cleavage had occurred during biosynthesis (G/S is the glycine↓serine cleavage site). We were suspicious, therefore, that cleavage of rMuc3 may require the presence of a more distant amino acid sequence on the C-terminal side of the G/SIVV site. Addressing this question, and thus determining the functional importance of the entire SEA module for cleavage, was one of the goals of the present study.

The product of another rMuc3 construct (p20s/a) in which the G/S-cleavage-site serine residue was mutated to alanine, failed to produce proteolytic fragments and was not secreted [10]. Whether this uncleaved product was correctly processed and targeted to the plasma membrane was not investigated. An attempt to establish a functional linkage between cleavage at the C-terminus and membrane targeting of the mucin represented a second goal of the present study.

In well characterized proteins containing SEA modules, it has been noted that the proteins act in heavily O-glycosylated surroundings [13]. In mucins, the regions which actually comprise the SEA modules are not heavily O-glycosylated, but contain a limited number of N-glycan consensus sites. The possibility that N-glycans might govern folding, cleavage, re-association of fragments, or membrane targeting of rMuc3 thus represented a third major goal of the present study.

Our findings provide a better understanding of the factors regulating membrane-mucin SEA-module cleavage, post-cleavage association, cell transport and membrane targeting.

MATERIALS AND METHODS

Reagents

Cell labelling ^{35}S-Pro-mix containing L-[^{35}S]methionine and L-[^{35}S]cysteine was purchased from Amersham Corp (Oakville, Ontario, Canada). Protein A–agarose, and tunicamycin were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (N-glycosidase F) (EC 3.2.1.96) was from Roche Molecular Biochemicals (Laval, Quebec, Canada). Monoclonal anti-Myc and anti-(V5 epitope) antibodies were obtained from Invitrogen (Groningen, The Netherlands). His-Bind® resin and buffer kit were purchased from Novagen Inc. (Madison, WI, U.S.A.). Anti-6279 antibody was developed against a peptide sequence in the C-terminal domain of rat Muc3, and has been described previously [10].

C-terminal domain constructs of rMuc3 (p20, p20t, p20s/a and p20SEA)

Constructs p20, p20t and p20s/a and the pSec expression vector used in these studies have been described previously [10] and are shown in Figure 1. Another construct, p20SEA, was produced by site-specific mutation of p20 using the Quickchange™ site-directed mutagenesis kit (Stratagene). A stop codon was introduced at amino acid position 228 to produce a truncated form lacking the second EGF motif, transmembrane region (TM) and the cytoplasmic tail.

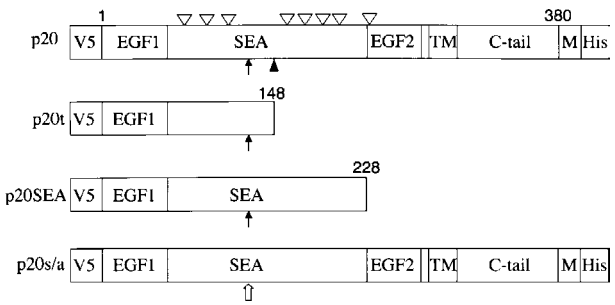

Figure 1 Schematic representation of rMuc3 constructs

cDNA (p20) for the C-terminal 380 amino acids of rMuc3 was cloned into the expression vector pSec. 'EGF'1, 'EGF2', 'SEA' and 'TM' represent two EGF-like domains, the SEA module and TM region respectively. 'V5', 'M' and 'His' refer to V5, Myc and polyhistidine tags. Open arrowheads (▽) indicate N-glycan consensus sites. Closed arrowhead (▲) indicates the approximate position of the Muc3 epitope designated 6279. The arrow (↑) points to the G/S cleavage site (amino acid position 125) in the LSKGSIVV sequence. Truncated mutants include p20t and p20SEA, and p20s/a refers to a p20 construct containing serine-to-alanine mutation at the G/S cleavage site (⇒).

The primer pair used for PCR was sense 5′-CTGTGTCACGCCT**TAA**TGTTCATCAGGC-3′ and antisense 5′-GCCTGATGAACA**TTA**AGGCGTGACACA-3′. Nucleotides shown in **bold** typeface represent the stop codon. Mutations were confirmed by DNA sequencing (Biotechnology Service Center, The Hospital for Sick Children, Toronto, Canada).

Transfection experiments

DNA transfections in COS-1 cells, SDS/PAGE and Western blotting of lysates and spent media were done as described previously [10]. Since the products of p20t and p20SEA were mostly secreted, assays of media rather than cell lysates for these samples were often chosen for presentation in the results (see below).

Purification and deglycosylation of His-tagged proteins with N-glycosidase F

His-tagged products of cell lysates were purified on a His*Bind® resin (Novagen, Madison, WI, U.S.A.), boiled for 5 min in 1 % SDS, incubated at 37 °C for 16 h with or without 20 units of N-glycosidase F in 10 mM phosphate buffer, pH 7.0, containing 30 mM sodium EDTA and 0.7 % Nonidet P40. At the end of the incubation period, an aliquot was concentrated and subjected to SDS/PAGE and Western blotting using anti-V5 antibody or anti-6279 antibody (epitope indicated in Figure 1 by an arrowhead). In the case of p20t and p20SEA transfections, N-glycosidase F (10 units) was added directly to the spent medium and incubated for 16 h at 37 °C prior to SDS/PAGE.

Metabolic labelling and immunoprecipitation of expressed proteins

^{35}S pulse–chase experiments with transfected cells and immunoprecipitation of products with anti-V5 antibody were carried out as described previously [10]. In some experiments, transfected cells

were also incubated in the presence of tunicamycin (20 μg/ml) 2 h prior to the radioactive pulse, and tunicamycin was maintained throughout the entire pulse (15 min) and chase (0–4 h) periods. In the case of tunicamycin-treated p20SEA transfections, an aliquot of spent medium was also treated with N-glycosidase F prior to immunoprecipitation.

Immunolocalization of expressed proteins in transfected cells

COS-1 cells were grown in square mini plastic plates (10 mm × 10 mm) and transfected with p20, p20s/a or p20t. In some experiments, transfected cells were incubated with 5 μg/ml tunicamycin for the last 16 h of transfection. Following rinsing with PBS, cells were processed for immunolocalization with or without prior fixing for 5 min in chilled methanol. Non-specific binding was blocked by incubating cells for 1 h at 22 °C with 5 % normal goat serum in PBS. Immune serum binding was carried out by incubating overnight at 4 °C with anti-V5 or anti-Myc antibodies at dilutions of 1:200 (v/v). Cells were rinsed five times with PBS and incubated with cyanine (Cy^3) fluorophore-tagged goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Mississauga, ON, Canada) for 1 h at 22 °C. Following five washes with PBS, cells were counterstained with Mayer's haematoxylin, 'coverslipped' using VectaShield mounting media (Vector Laboratories, Burlington, CA, U.S.A.), and examined under dark-field microscopy and photographed. Images were scanned and converted into black-and-white images by use of Adobe Photoshop software.

Denaturation by SDS or heating

Prior to immunoprecipitation, ^{35}S-labelled cell lysates of p20 were subjected to 2 % (w/v) SDS or boiling for 5 min. Samples containing 2 % SDS were diluted with radioimmunoprecipitation buffer ('RIPA' [10]) and the SDS concentration was decreased to 0.4 %. Boiled samples were cooled to room temperature (20 °C). Immunoprecipitation was then carried out as described previously [10] using anti-V5 antibody.

RESULTS

Cleavage of rMuc3 at the G/SIVV site requires the presence of a more C-terminally located sequence in the SEA module

The four constructs used in the present study are shown in Figure 1. The sequence for the C-terminal 380 amino acids of rMuc3 is encoded by p20. In our previous studies we established that this product is cleaved at the G/SIVV sequence (arrow) resulting in a V5-positive N-terminal fragment and a Myc-positive C-terminal fragment. As shown in Figure 2(A), Western blots of p20 products showed V5-positive bands at 30 and 27 kDa (lane 1). Following treatment with N-glycosidase F, these were reduced to a single band at 22 kDa (lane 2). The C-terminal fragment (Figure 2B), appeared as a broad band most intense at about 49 kDa on Western blots with anti-Myc and anti-6279 antibodies (lanes 1 and 3). Following treatment with N-glycosidase F, the C-terminal product was reduced to a doublet at about 41 kDa, as judged by both anti-Myc and anti-6279 antibodies (Figure 2B, lanes 2 and 4).

Two other constructs, p20t and p20SEA, were designed to determine if sequences distant from the G/SIVV site were important for the cleavage event observed in the p20 product. As shown in Figure 1, the product of p20t is truncated in its C-terminal region such that it contains only the first 148 amino acids

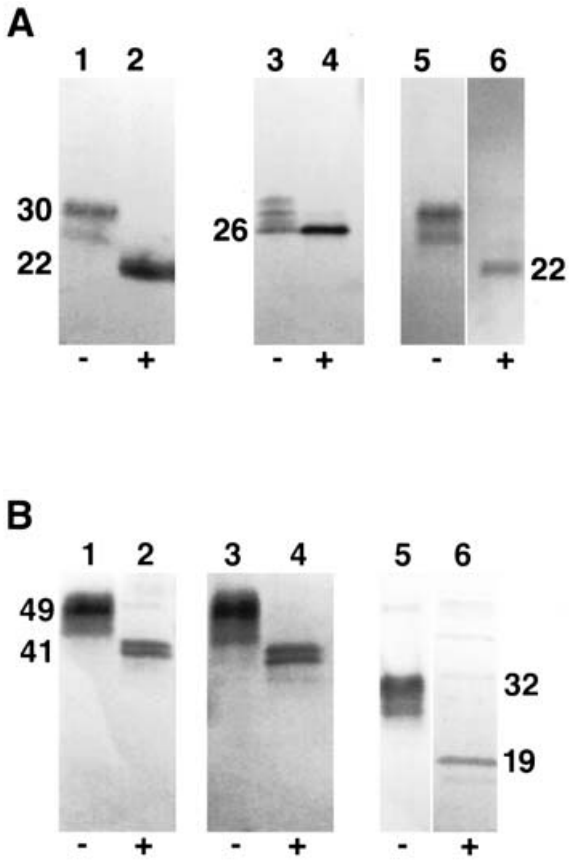

Figure 2 Expression of p20, p20t and p20SEA in COS-1 cells

His*Bind®-purified p20 products in transfected cell lysates (**A**, lanes 1 and 2, and **B**, lanes 1–4) and spent media of p20t (**A**, lanes 3 and 4) or p20SEA (**A** and **B**, lanes 5 and 6) were incubated with (+) or without (−) N-glycosidase F. Detection was carried out by SDS/PAGE and Western blotting with anti-V5 antibody (**A**), anti-Myc (**B**, lanes 1 and 2) and anti-6279 (**B**, lanes 3–6) antibodies. Numbers on either side represent molecular mass (in kDa).

of p20, which include the GSIVV sequence with the G/S cleavage site located at amino acid position 125 and an additional 22 amino acids beyond the cleavage site.

When the truncated p20t construct was expressed in COS-1 cells, the product was found mainly (67 %) in the medium, and was detected as a V5-positive ladder of glycoforms (range 26–38 kDa) (Figure 2A, lane 3). Following N-glycosidase F treatment, the bands collapsed to 26 rather than to 22 kDa (lane 4). If the p20t product had been cleaved at the G/SIVV site, the non-glycosylated V5-positive N-terminal fragment of p20t should have been at 22 kDa, as it was for p20. We conclude, therefore, that p20t was expressed, glycosylated and secreted, but was not cleaved at the G/SIVV site.

The rMuc3 product of p20SEA is also truncated in its C-terminal region, but has a total of 228 amino acids (Figure 1) and is therefore much longer than the p20t product. If cleavage occurs at the G/SIVV site, the V5-positive N-terminal fragment of p20SEA was expected to be identical with that of p20 (i.e. 22 kDa). However, the C-terminal fragment of p20SEA was expected to be shorter than that of p20 (Figure 1), since the EGF2-to-His segment is missing.

The p20SEA product was expressed, glycosylated and mainly (72 %) secreted, appearing as a V5-positive broad band at about 30 kDa (Figure 2A, lane 5) or a ladder of glycoforms ranging from 27 to 30 kDa. If no cleavage had occurred, the product would be

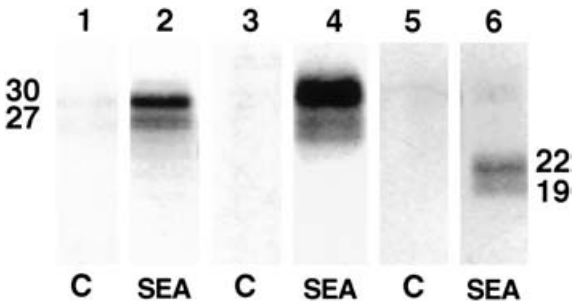

Figure 3 Immunoprecipitation of p20SEA products using anti-V5 antibody

COS-1 cells transfected with construct p20SEA ('SEA') were pulsed with [^{35}S]cysteine/[^{35}S]methionine for 15 min and chased for 4 h. Cell lysates (lanes 1 and 2) and spent medium (lanes 3–6) were immunoprecipitated with anti-V5 antibody, separated by SDS/PAGE and detected by autoradiography. 'C' refers to vector alone. In lanes 5 and 6, products were treated with N-glycosidase F prior to immunoprecipitation. Numbers on either side represent molecular mass (in kDa).

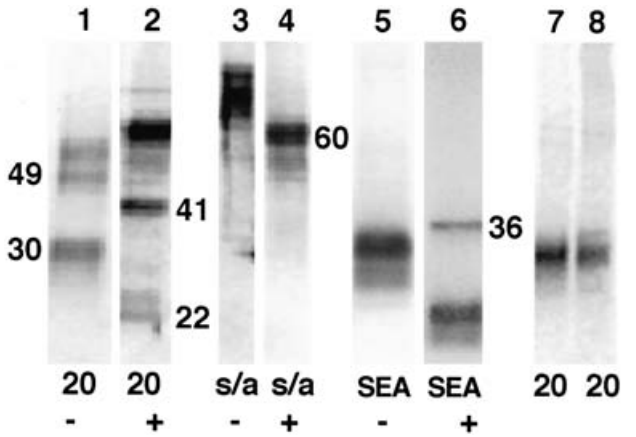

Figure 4 Effect of tunicamycin on products from p20, p20s/a and p20SEA transfections

COS-1 cells transfected with p20 ('20'), p20s/a ('s/a') or p20SEA ('SEA') were pulsed with [^{35}S]cysteine/[^{35}S]methionine for 30 min and chased for 4 h. Cell lysates (lanes 1–4) and spent media (lanes 5 and 6) were immunoprecipitated with anti-V5 antibody, separated by SDS/PAGE and detected by autoradiography. Symbols + and − represent treatment with or without tunicamycin. Lanes 7 and 8 represent cell lysates of p20 transfections treated with heat or 2 % SDS prior to immunoprecipitation. Numbers beside the lanes represent molecular mass (in kDa).

expected at a position of 45 kDa or more. Digestion of the p20SEA product (in spent medium) with N-glycosidase F gave rise to a V5 product at 22 kDa (Figure 2A, lane 6), indicating that the usual N-terminal cleavage fragment had indeed been generated in p20SEA transfects. The C-terminal fragment appeared as a broad band at about 32 kDa on Western blots using the anti-6279 antibody for detection (Figure 2B, lane 5). Following treatment with N-glycosidase F, the product was reduced to a band at 19 kDa (Figure 2B, lane 6). We conclude, therefore, that amino acids within the SEA module, but located a considerable distance from the G/S site (i.e. beyond the 22 amino acids C-terminal of the site, which were present in p20t), were needed for G/S cleavage to occur. Cleavage did not require the presence of more distant domains, such as the EGF2, TM or C-tail domains, since these were not present in the p20SEA construct.

The SEA module is sufficient for re-association of cleaved fragments

As shown previously, re-association of cleaved fragments occurs during biosynthesis shortly after the product of p20 is cleaved in the ER [10]. The re-association depends upon unidentified non-covalent interactions (i.e. independent of disulphide bonds). If the SEA-module sequence is sufficient to allow for re-association of the cleaved fragments, we expected that the two cleavage fragments of p20SEA transfectants would both be immunoprecipitated by the anti-V5 antibody. Pulse–chase studies were carried out using vector alone (control) and p20SEA constructs as shown in Figure 3. In the immunoprecipitates of both cell lysates (lane 2) and media (lane 4) the cleavage products were detected at positions corresponding to 30 and 27 kDa (i.e. the same as those seen earlier; Figure 2). The more diffuse bands seen in medium samples (lane 4) reflect the greater concentration of product in the spent medium. Both fragments were co-precipitated by anti-V5, thereby confirming that the SEA module is sufficient to permit re-association after cleavage. The major difference noted in the intensities of the two bands is probably a reflection of the number of cysteine/methionine residues present in each fragment. The N-terminal fragment of p20SEA has four times more residues available for labelling with [^{35}S]cysteine/[^{35}S]methionine labelling than the C-terminal fragment.

An aliquot of spent media from p20SEA transfection was also subjected to N-glycosidase F prior to immunoprecipitation with anti-V5 to determine whether N-glycans present on the fragments were needed to maintain the association of the two fragments. This was clearly not the case, because the two non-glycosylated fragments of p20SEA cleavage (22 and 19 kDa) were co-precipitated by anti-V5 antibody (lane 6). Thus the non-covalent interactions which stabilize re-association of the cleavage fragments do not depend on the presence of N-linked oligosaccharides.

Heating and/or SDS prevent re-association of rMuc3 cleavage fragments

Following transfection of COS-1 cells with p20, cells were pulse-labelled and chased as described above. At the end of chase period (4 h), cell lysates were either subjected to 2 % SDS treatment or boiling (100 °C for 5 min) prior to immunoprecipitation with anti-V5 antibody. As shown in Figure 4 (lanes 7 and 8), compared with untreated cell-lysate controls (lane 1), anti-V5 antibody immunoprecipitated the N-terminal 30 kDa fragment but failed to precipitate the 49 kDa C-terminal fragment. Thus pre-boiling or preincubation in 2 % SDS prior to immunoprecipitation prevented re-association of the 30 and 49 kDa fragments. Furthermore, once dissociated, the fragments failed to re-associate, despite removal of SDS (dilution to 0.4 %) and/or cooling of the samples to room temperature.

N-linked oligosaccharides influence the cleavage reaction

In pulse–chase experiments, tunicamycin (20 μg/ml) was added for 2 h to cells transfected with wild-type p20 or its counterpart p20s/a in which the cleavage site G/S was mutated. Cell lysates and media were immunoprecipitated with anti-V5 antibody and the labelled products analysed by SDS/PAGE and autoradiography (Figure 4). In the absence of tunicamycin, p20 constructs expressed the usual laddered 49 and 30 kDa fragments (lane 1), while cells grown in the presence of added tunicamycin produced bands at 60, 41 and 22 kDa (lane 2). The 41 and 22 kDa

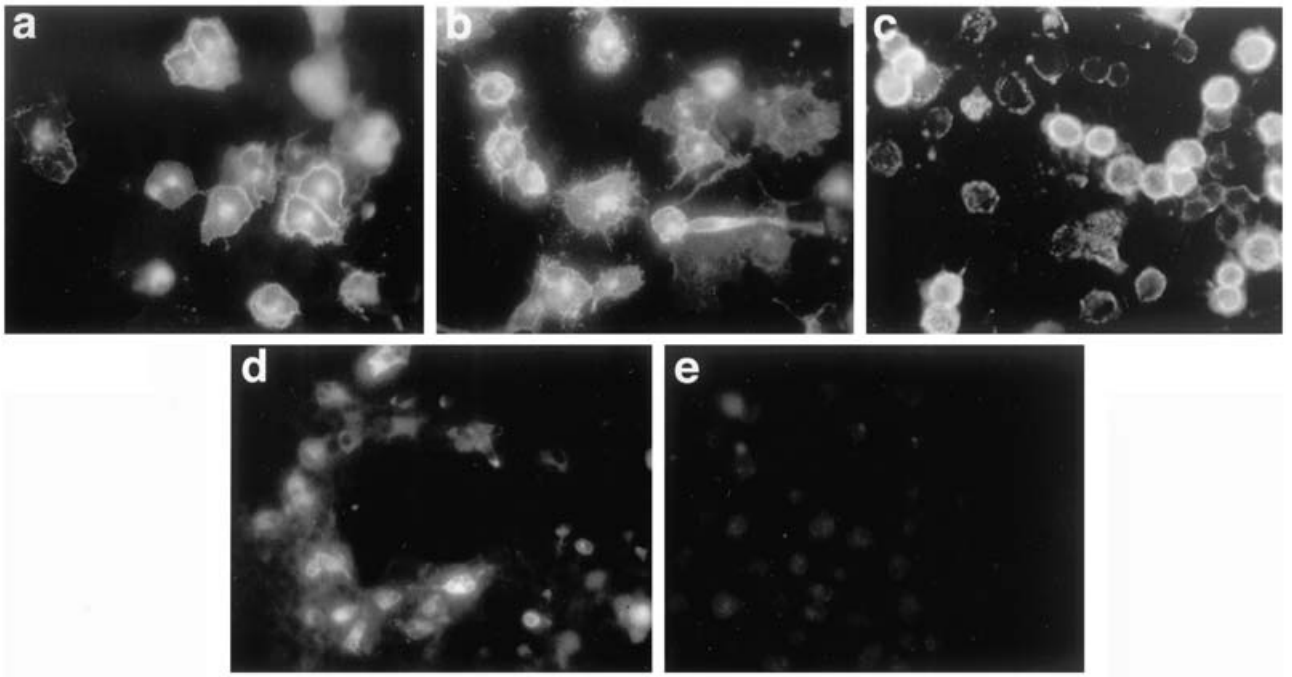

Figure 5 Immunolocalization of rMuc3 products of p20 and p20t

Immunolocalization was carried out on COS-1 cells transfected with p20 (**a**–**c**) or p20t (**d** and **e**) using anti-V5 antibody (**a**, **c**, **d** and **e**) or anti-Myc antibody (**b**). In (**a**), (**b**) and (**d**), cells were fixed in methanol, whereas in (**c**) and (**e**), cells were not fixed. Magnification 400×.

bands represent the non-glycosylated fragments of p20 cleavage, corresponding to those generated earlier by N-glycosidase F (Figure 2). In the absence of tunicamycin, p20SEA constructs expressed products at 30 and 27 kDa (lane 5), while cells grown in the presence of tunicamycin expressed bands at 22 and 19 kDa (lane 6), also as expected from results produced previously and shown in Figure 3. Thus the lack of N-glycans in the rMuc3 constructs did not prevent either cleavage or re-association of cleavage fragments. However, in both transfectants, tunicamycin had an additional effect, which was to cause some of the non-cleaved, non-glycosylated product to accumulate. The relevant band was seen at 60 kDa in the case of p20 (lane 2) and at 36 kDa in the case of p20SEA (lane 6). The identification of 60 kDa as the non-cleaved non-glycosylated product of p20 was supported by its recognition by both anti-V5 and anti-Myc antibodies (the latter not presented), and by its correspondence with the mobility of the non-glycosylated product of the p20 s/a construct (lane 4). Thus tunicamycin caused a partial inhibition of rMuc3 domain cleavage. Because tunicamycin exerts widespread effects to prevent N-linked glycosylation of all or most glycoconjugates in cells, the partial inhibition of cleavage of p20 and p20SEA cannot be ascribed necessarily to a lack of N-glycans on the rMuc3 polypeptide. Nevertheless the results leave open this possibility and the notion, therefore, that N-glycans on rMuc3 somehow directly facilitate ER cleavage at the G/SIVV site. Further research is required to explore this hypothesis.

Targeting of rMuc3 products to the cell membrane

Influences governing membrane targeting were explored by immunolocalization experiments using both fixed (permeabilized) and non-fixed (non-permeabilized) COS-1 cells after transfection. The importance of the TM plus C-tail region was investigated by comparing V5 and Myc immunoreactivity in p20 and p20t transfectants. As shown in Figures 5(a) and 5(b), the product of p20 was detected by both antibodies in fixed cells in a perinuclear position and on the cell surface. In non-permeabilized cells, immunostaining was confined to the cell surface (Figure 5c). Vector-alone (control) transfectants were negative (not presented). Thus despite cleavage of the p20 product and re-association of cleavage fragments, the putative 'soluble' extracellular fragment remained anchored to the cell surface with its N-terminal V5 epitope exposed. The expressed product of p20t as detected by anti-V5 antibody was localized only around the perinuclear region, as observed in a few of the permeabilized cells (Figure 5d), and no staining was observed in non-permeabilized cells (Figure 5e). Both results are consistent with previously obtained results showing that most of the p20t product is secreted.

To determine whether cleavage of the SEA module and re-association of fragments were needed for membrane targeting, the V5 immunoreactivity of p20 and p20s/a transfectants were compared. In Figure 6, the distribution of fluorescence in both fixed (Figure 6a) and non-fixed (Figure 6b) p20s/a transfectants resembled that of p20 transfectants (Figures 5a and 5c), in showing staining both inside and on the surface of cells. Thus cleavage was not essential to obtain correct membrane targeting of rMuc3.

The possible requirement for N-glycosylation of rMuc3 (or its cleavage fragments) to obtain membrane targeting was investigated by comparing V5 immunoreactivity of p20 and p20s/a transfectants preincubated with or without tunicamycin (5 μg/ml for 16 h). Non-permeabilized cells, shown in Figures 6(c) and 6(d), revealed that membrane targeting of the rMuc3 domain occurs even in cells treated with tunicamycin.

Immunolocalization experiments thus indicate that the presence of the TM plus C-tail region is essential for membrane targeting, but neither cleavage in the SEA module nor glycosylation regulate membrane targeting.

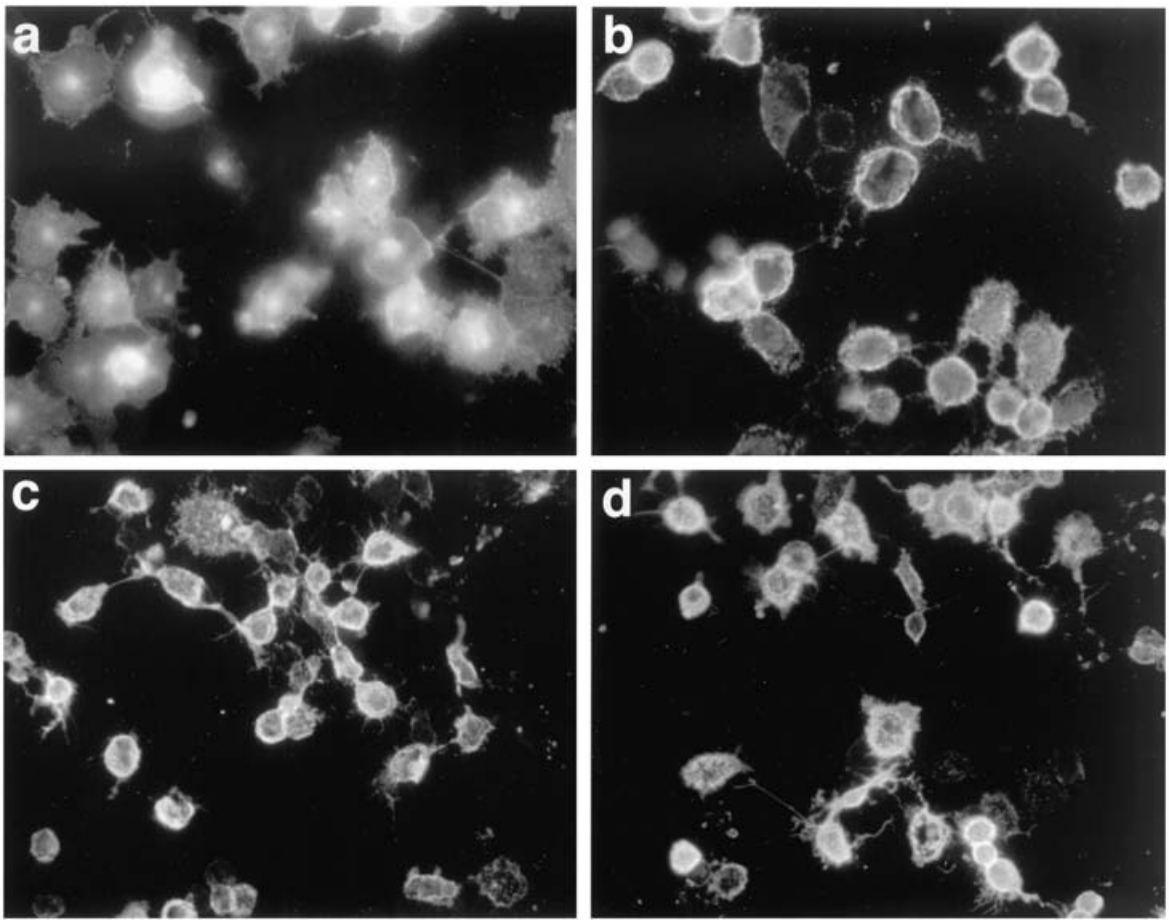

Figure 6 Immunolocalization of p20 and p20s/a products in cells incubated with tunicamycin

COS-1 cells transfected with p20s/a (**a**, **b** and **d**) or p20 (**c**) were incubated in the presence (**c** and **d**) or absence (**a** and **b**) of tunicamycin. Immunolocalization was carried out using anti-V5 antibody. Cells were either not fixed (**b**, **c** and **d**) or fixed in methanol (**a**). Magnification: (**a**) and (**b**), 400×; (**c**) and (**d**), 250×.

DISCUSSION

SEA modules in mucins

Membrane mucins have now been added to the growing list of diverse O-glycosylated proteins that contain SEA modules. Although sequence homology is not prominent among SEA modules, the presence of a conserved proteolytic consensus sequence (G/SVVV) within an 80–110-residue domain of alternating α-helix and β-sheet secondary structure are defining characteristics. Thus SEA modules of different proteins are structurally homologous domains. It should be noted that one of the best-studied membrane-associated mucins, namely rat Muc4 (formerly called 'asialoglycoprotein' or 'ASGP'), does not contain a SEA module, but undergoes proteolytic cleavage at a different (aspartic acid↓proline) target site, with non-covalent re-association of fragments (heterodimeric subunits) and involvement in many signal transduction-mediated events [3,19]. Thus SEA modules, although important, may not be the only motif used by surface mucins to allow cleavage and to initiate a signalling cascade.

Cleavage of the core peptide of MUC1 near the C-terminus was first described by Ligtenberg et al. [20] and localized to a stretch of residues N-terminal to the transmembrane segment. The specific cleavage site (G/SVVV) was later identified by Parry et al. [17], and both groups confirmed that the cleaved fragments remain associated after cleavage by non-covalent bonds. The location of this site within a SEA module and the existence of several MUC1 mutants and splice variants, led Wreschner et al. [15] to test whether there were specific SEA-module sequence requirements for cleavage and, in particular, for re-association of the cleaved fragments. One cluster (-43 to -59 residues N-terminal of the G/S site near the N-terminal border of the SEA module) was needed for cleavage, while several other clusters (-1 to -4, -10 to -21, -28 to -32 and $+1$ to $+12$, where 0 represents the serine residue of the G/S site) were determined as needed for re-association of the fragments. Because of the lack of sequence homology among SEA modules, these clusters cannot be extrapolated to other mucins as general requirements for SEA module function. However, the results suggest as a minimum that binding sequences located in regions remote from the G/S target site may govern the efficiency of cleavage or re-association of fragments or both.

Factors influencing cleavage in the C-terminal SEA module of rMuc3

The results of our studies indicate that early cleavage of rMuc3 requires the presence of the target G/SIVV site and sequences distant from it. The truncated p20t product, which includes 22 amino acids C-terminal of the site, did not get cleaved, whereas

longer products (such as p20) did. The p20SEA product was also cleaved, indicating that the presence of EGF2, and domains further C-terminal to it, were not required for cleavage. Since MUC1 is cleaved efficiently and yet contains no EGF regions, it is likely that EGF regions are not needed for cleavage of rMuc3 or any other membrane mucins.

The possibility that N-glycans in rMuc3 could have an influence on cleavage was investigated in the present study, since N-glycosylation can exert profound effects on polypeptide folding and stability [21]. We wondered if failure of post- or co-translational N-glycosylation might prevent accessibility of the cleavage enzyme to the target G/S site. Despite tunicamycin treatment of cells, however, cleavage of the SEA module proceeded. In the same experiments it was noted that some non-cleaved non-glycosylated product accumulated. The lack of complete cleavage may or may not be a direct effect of inhibition of rMuc3 N-glycosylation, since tunicamycin is expected to have widespread effects on many other cell glycoconjugates, including putative cleavage enzymes. However, our findings raise the possibility that efficient cleavage may depend on the correct three-dimensional configuration of the SEA module, which, in turn, could be influenced by ER-mediated N-glycosylation. Suitable N-glycan site mutants will need to be constructed to test this hypothesis.

Factors influencing re-association of rMuc3 cleavage products

N-glycosylation was not required for re-association of rMuc3 cleaved fragments, since the two were co-immunoprecipitated by anti-V5 antibody in cells previously treated with tunicamycin. The same was true if the products were deglycosylated by N-glycosidase F before immunoprecipitation (Figure 3, lane 6). Re-association of the fragments was, however, prevented by SDS (2 %) or by boiling of samples, indicating that strong non-covalent interactions are responsible for holding the fragments together. All of the structural and/or sequence information required to promote re-association probably lies within the peptide backbone of the SEA module, since construct p20SEA (which lacks EGF2 and further C-terminal domains) exhibited re-association of the cleaved fragments.

Factors influencing membrane targeting of rMuc3

Although many membrane mucins are likely to undergo SEA-module cleavage, there are no studies to date which establish whether cleavage is a requirement for correct targeting of a mucin to its final destination in the plasma membrane. For rMuc3, cleavage was not a prerequisite, since the expressed p20s/a mutant, which is not cleaved, was nevertheless immunolocalized to the plasma membrane. N-glycosylation was also not required, since tunicamycin-treated cells still permitted surface-membrane targeting of the non-glycosylated fragments. The only apparent requirement for membrane targeting was the presence of domains on the C-terminal side of the SEA module (presumably the TM and/or C-tail segment). Without these, as in the p20t or p20SEA constructs, the expressed products were not on the plasma membrane, but were found chiefly in the medium.

The cleavage endoproteinase

The enzyme(s) responsible for SEA-module cleavage at the G/SVVV site has not been identified. It doubtless resides in the ER compartment, since cleavage occurs within 15 min of protein synthesis [10]. Further, the responsible enzyme(s) is (are) likely to be ubiquitous, since proteins containing SEA modules are found in cells all over the body. Parry et al. [17] have suggested that kallikrein-like proteinases may be responsible, but no investigations to identify specific enzymes have been reported to date.

Implications

It is very likely that membrane mucin cleavage fragments function generally in a ligand-receptor partnership as suggested earlier [15], to regulate intracellular signal transduction and thereby play a role in diverse processes such as growth, differentiation, cell–cell, or cell–bacterial interactions, immune cell recognition and/or metastases. Considerable evidence has been reported to support this hypothesis using MUC1, MUC4 and rat Muc4 as models of membrane mucins [1,3,22,23]. Signal transduction is mediated by phosphorylation of tyrosine (or serine) residues in the C-tail domain [2]. Judging from the results of the present study, and the presence of a tyrosine phosphorylation consensus sequence in the C-tail [24], rMuc3 will also serve as a useful model in intestinal cells. Knowledge of the factors governing cleavage and re-association of membrane-mucin segments may be expected eventually to lead to the design of specific blocking agents which could prevent or modify deleterious effects of mucin-induced signalling in human diseases such as cancer, bacterial infection or inflammatory bowel disease.

Financial support for this study was received from the Canadian Cystic Fibrosis Foundation, the Canadian Institutes for Health Research and the Hospital for Sick Children, Toronto, ON, Canada.

REFERENCES

1 McNeer, R. R., Huang, D., Fregien, N. L. and Carraway, K. L. (1998) Sialomucin complex in the rat respiratory tract: a model for its role in epithelial protection. Biochem. J. **330**, 737–744

2 Baruch, A., Hartmann, M., Yoeli, M., Adereth, Y., Greenstein, S., Stadler, Y., Skornik, Y., Zaretsky, J., Smorodinsky, N. I., Keydar, I. and Wreschner, D. H. (1999) The breast cancer-associated MUC1 gene generates both a receptor and its cognate binding protein. Cancer Res. **59**, 1552–1561

3 Carraway, K. L., Price-Schiavi, S. A., Komatsu, M., Idris, N., Perez, A., Li, P., Jepson, S., Zhu, X., Carvajal, M. E. and Carraway, C. A. C. (2000) Multiple facets of sialomucin complex/MUC4, a membrane mucin and erbB2 ligand, in tumors and tissues. Front. Biosci. **5**, d95–d107

4 Schroeder, J. A., Thompson, M. C., Gardner, M. M. and Gendler, S. J. (2001) Transgenic MUC1 interacts with epidermal growth factor receptor and correlates with mitogen-activated protein kinase activation in the mouse mammary gland. J. Biol. Chem. **276**, 13057–13064

5 Smorodinsky, N., Weiss, M., Hartmann, M. L., Baruch, A., Harness, E., Yaakobovitz, M., Keydar, I. and Wreschner, D. H. (1996) Detection of a secreted MUC1/SEC protein by MUC1 isoform specific monoclonal antibodies. Biochem. Biophys. Res. Commun. **228**, 115–121

6 Shirazi, T., Longman, R. J., Corfield, A. P. and Probert, C. S. J. (2000) Mucins and inflammatory bowel disease. Postgrad. Med. J. **76**, 473–478

7 Buisine, M. P., Desreumaux, P., Leteurtre, E., Copin, M. C., Colombel, J. F., Porchet, N. and Aubert, J. P. (2001) Mucin gene expression in epithelial cells in Crohn's disease. Gut **49**, 544–551

8 Khatri, I. A., Ho, C., Specian, R. D. and Forstner, J. F. (2001) Characteristics of rodent intestinal mucin Muc3 and alterations in a mouse model of human cystic fibrosis. Am. J. Physiol. Gastrointest. Liver Physiol. **280**, G1321–G1330

9 Parmley, R. R. and Gendler, S. J. (1998) Cystic fibrosis mice lacking Muc1 have reduced amounts of intestinal mucus. J. Clin. Invest. **102**, 1798–1806

10 Wang, R., Khatri, I. A. and Forstner, J. F. (2002) The carboxyl terminal domain of rodent intestinal mucin Muc3 is proteolytically cleaved in the endoplasmic recticulum to generate extracellular and membrane components. Biochem. J. **366**, 623–631

11 Julian, J. and Carson, D. D. (2002) Formation of MUC1 metabolic complex is conserved in tumor-derived and normal epithelial cells. Biochem. Biophys. Res. Commun. **293**, 1183–1190

12 Carraway, K. L. I., Rossi, E. A., Komatsu, M., Price-Schiavi, S. A., Huang, D., Guy, P. M., Carvajal, M. E., Fregien, N., Carraway, C. A. C. and Carraway, K. L. (1999) An intramembrane modulator of the erbB2 receptor tyrosine kinase that potentiates neuregulin signaling. J. Biol. Chem. **274**, 5263–5266

13 Bork, P. and Patthy, L. (1995) The SEA module: a new extracellular domain associated with O-glycosylation. Protein Sci. **4**, 1421–1425
14 Stacey, M., Lin, H., Gordon, S. and McKnight, A. J. (2000) LNB–TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors. Trends Biochem. Sci. **25**, 284–289
15 Wreschner, D. H., McGuckin, M. A., Williams, S. J., Baruch, A., Yoeli, M., Ziv, R., Okun, L., Zaretsky, J., Smorodinsky, N., Keydar, I. et al. (2002) Generation of ligand-receptor alliances by "SEA" module-mediated cleavage of membrane-associated mucin proteins. Protein Sci. **11**, 698–706
16 Gum, J. R. J., Crawley, S. C., Hicks, J. W., Szymkowski, D. E. and Kim, Y. S. (2002) MUC17, a novel membrane-tethered mucin. Biochem. Biophys. Res. Commun. **291**, 466–475
17 Parry, S., Silverman, H. S., McDermott, K., Willis, A., Hollingsworth, M. A. and Harris, A. (2001) Identification of MUC1 proteolytic cleavages sites *in vivo*. Biochem. Biophys. Res. Commun. **283**, 715–720
18 Abe, J., Fukuzawa, T. and Hirose, S. (2002) Processing and subunit structure of Ig-Hepta, a seven-transmembrane receptor with a Long N-terminal extracellular domain. J. Biol. Chem. **277**, 23391–23398
19 Sheng, Z., Wu, K., Carraway, K. L. and Fregien, N. (1992) Molecular cloning of the transmembrane component of the 13762 mammary adenocarcinoma sialomucin complex: A new member of the epidermal growth factor superfamily. J. Biol. Chem. **267**, 16341–16346
20 Ligtenberg, M. J. L., Kruijshaar, L., Buijs, F., Meijer, M. v., Litvinov, S. V. and Hilkens, J. (1992) Cell-associated episialin is a complex containing two proteins derived from a common precursor. J. Biol. Chem. **267**, 6171–6177
21 Satyanarayana, J., Gururaja, T. L., Narasimhamurthy, S., Naganagowda, G. A. and Levine, M. J. (2001) Synthesis and conformational features of human salivary mucin C-terminal derived peptide epitope carrying Thomsen–Friedenreich antigen: implications for its role in self-association. Biopolymers **58**, 500–510
22 Hanisch, F. and Muller, S. (2000) MUC1: the polymorphic appearance of a human mucin. Glycobiology **10**, 439–449
23 Carraway, K. L., Ramsauer, V. P., Haq, B. and Carraway, C. A. C. (2002) Cell signalling through membrane mucins. BioEssays **25**, 66–71
24 Khatri, I. A., Forstner, G. G. and Forstner, J. F. (1997) The carboxyl-terminal sequence of rat intestinal mucin RMuc3 contains a putative transmembrane region and two EGF-like motif. Biochim. Biophys. Acta **1326**, 7–11

Received 22 August 2002/20 February 2003; accepted 27 February 2003
Published as BJ Immediate Publication 27 February 2003, DOI 10.1042/BJ20021333