Synthesis and secretion of Muc4/sialomucin complex: implication of intracellular proteolysis

Masanobu KOMATSU, Maria E. ARANGO and Kermit L. CARRAWAY¹

Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, FL 33101, U.S.A.

Muc4/sialomucin complex (SMC) is a heterodimeric glycoprotein complex implicated in epithelial protection and overexpressed in some tumours. It is encoded by a single gene, and the two subunits are produced by proteolytic cleavage at a time before substantial O-glycosylation, near the time of transit from the endoplasmic reticulum to the Golgi. Although Muc4/SMC is translated as a membrane protein, it is produced as a soluble form in many epithelia. Transfection experiments using Cos-7, HBL-100 human epithelial, MCF-7 human breast tumour and HC11 mouse mammary cell lines showed that soluble rat Muc4/SMC is produced by a proteolytic cleavage mechanism and released by secretion from multiple cell lines, including both human and mouse normal epithelial cells and tumour cells. Similar transfection experiments demonstrated the same mechanism for the human analogue MUC4. Gel electrophoresis analyses of deglycosylated membrane and soluble trans-

membrane subunits and of the membrane-associated cleavage fragment indicated a released cleavage product of 25 kDa, resulting from cleavage between two epidermal growth factor-like domains. Further evidence for this site was obtained from deletion mutants removing this region of the protein, which blocked secretion. Finally, pulse-chase analyses of Muc4/SMC biosynthesis indicated no kinetic difference between the timing of the cleavage to release the soluble form and that to produce the two subunits, indicating that the soluble form is created early in transit to the cell surface. These studies provide the first clear evidence that membrane mucins can be released from cells by an intracellular proteolytic mechanism that leads to secretion of the soluble form of the mucin.

Key words: biosynthesis, membrane, mucin, processing, transfection.

INTRODUCTION

MUC4 is one of the membrane mucins of the human mucin gene (*MUC*) family. The rat homologue of human MUC4, rat Muc4, was isolated originally from the highly malignant ascites 13762 rat mammary adenocarcinoma cells and called sialomucin complex (SMC) [1], and is herein designated Muc4/SMC. The human counterpart of this gene was partially cloned and sequenced from a tracheal cDNA library [2], then later fully cloned and sequenced to reveal the relationship to the rat Muc4/SMC [3]. Human MUC4 contains two tandem repeat sequences [3]. The one originally cloned is absent from the rat protein [3]. Muc4/SMC is encoded by a single gene and post-translationally processed into two subunits, ascites sialoglycoprotein (ASGP)-1 and ASGP-2 [1,4]. Heavily O-glycosylated mucin subunit ASGP-1 has a typical mucin-type rigid and extended structure [5]. The transmembrane subunit ASGP-2, which anchors the complex to the plasma membrane, contains two epidermal growth factor (EGF)-like domains, EGF-1 and EGF-2 [6]. EGF-1 can act as a ligand for the receptor tyrosine kinase ErbB2 [7]. Since Muc4/SMC is a heterodimeric complex derived from a single gene, the two subunits must be produced by proteolytic cleavage at some stage of their processing. Pulse labelling and immunoprecipitation studies indicate that both anti-ASGP-2 and the antibody against deglycosylated ASGP-1 recognize a 300 kDa N-glycosylated precursor in 13762 ascites tumour cells [4]. Pulse-chase analyses also show that the precursor is cleaved to yield ASGP-2 well before it reaches the cell surface [4]. Moreover, analyses with anti-ASGP-1 indicate that

the cleavage occurs before O-glycosylation of the mucin subunit, which happens near the time of transit from the endoplasmic reticulum to the Golgi [5]. Maturation of ASGP-1 by O-glycosylation was demonstrated by lectin precipitation and gel electrophoresis [4].

The function of this membrane mucin has been studied using rat Muc4/SMC cDNA isolated from ascites tumour cells [8]. Recent studies have revealed the multifunctionality of the glycoprotein, which can play a vital role in tumour progression and metastasis. As a strong anti-adhesive/anti-recognition agent, Muc4/SMC represses tumour cell adhesion to promote tumour dissemination and inhibit tumour cell destruction by host immune cells [8,9]. When overexpressed in carcinomas, Muc4/SMC accelerates local tumour growth [10] and substantially increases the incidence of distant metastasis [11]. Muc4/SMC is also an anti-apoptotic factor. Its expression reduces programmed cell death and promotes tumour survival [10]. The anti-apoptotic function of Muc4/SMC may be attributed, at least in part, to its ability to act as an intramembrane ligand for ErbB2 and to modulate tyrosine phosphorylation of ErbB2 via interaction of its EGF-like domain(s) with the receptor tyrosine kinase [7,12].

In normal epithelia, Muc4/SMC is proposed to provide a protective mechanism for vulnerable epithelial surfaces, such as those of the airway [13], female reproductive tract [14] and intestine [15]. Muc4/SMC is also produced by some glandular epithelia, such as the lacrimal [16], salivary [17] and mammary [15] glands. The Muc4/SMC level is approx. 100-fold less in lactating rat mammary gland than in the 13762 ascites tumour from which it was originally isolated [15]. Furthermore, ex-

Abbreviations used: ASGP, ascites sialoglycoprotein; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ITS, insulin, transferrin and sodium selenite; mAb, monoclonal antibody; SMC, sialomucin complex.

¹ To whom correspondence should be addressed, at Department of Cell Biology and Anatomy (R-124), University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33101, U.S.A. (e-mail kcarrawa@med.miami.edu).

pression in the virgin gland is about 100-fold lower than in the lactating gland [15]. Thus Muc4/SMC expression is up-regulated in mammary gland during pregnancy [18] and in some tumours [5]. Muc4/SMC expression on the apical surface of the rat uterine lining is regulated at the transcript level during pregnancy and the oestrous cycle [14], generating a barrier to blastocyst implantation during the non-receptive state [14]. Immunolocalization studies indicate that Muc4/SMC is present in the airway [13], female reproductive tract [14] and mammary gland [15] at the apical surfaces of epithelial cells. Muc4/SMC production is not limited to simple epithelia. Stratified epithelia of the cornea [19], conjunctiva [19], cervix [14], vagina [14] and oral cavity [20] also exhibit Muc4/SMC expression. The location in stratified epithelia varies with the particular tissue and its physiological state. For instance, Muc4/SMC locations in the stratified epithelia of the cervix and vagina are sensitive to the oestrous cycle of the rat [14].

Mucin glycoproteins of the MUC family can be classified as either integral membrane type or secreted/soluble type [21], based on the presence of a transmembrane sequence [22]. However, most, if not all, membrane mucins can also be produced as a soluble form [5]. Importantly, Muc4/SMC in normal human and rat tissues is present in both membrane-bound and soluble forms [23]. For instance, in lacrimal and mammary glands the membrane-bound Muc4/SMC is expressed as a soluble form that is secreted abundantly into tear fluid [16] and milk [15], respectively. Although most secretory epithelia expressing Muc4/SMC produce both forms, the relative abundance of each form depends on the tissue type [23], presumably indicating differential physiological roles of each form in each tissue. Presently, it is not known how the soluble form is produced by these cells. The ability of secretagogues to induce release of Muc4/SMC from rat colon, which produces primarily soluble form, suggest a secretion mechanism rather than shedding from the cell surface [15]. Attempts to demonstrate alternatively spliced Muc4/SMC in rat mammary gland and colon were unsuccessful [15], though evidence for alternative splicing of human Muc4/SMC has been presented [24]. In this paper, we explored the possible secretion mechanism using various mammalian cell lines transfected with rat Muc4/SMC and human MUC4 cDNAs. The expression of the cDNA encoding integral membrane Muc4/SMC revealed that the soluble form can be produced by a specific proteolytic cleavage of the membrane precursor during an early stage of Muc4/SMC biosynthesis. These studies demonstrated for the first time a proteolytic mechanism for Muc4/SMC secretion.

EXPERIMENTAL

Generation of cDNA constructs and transfection

Generation of cDNAs for Muc4/SMC analogues rep5 and rep1 was described previously [8]. These cDNAs were subcloned into pcDNA3 (Invitrogen, San Diego, CA, U.S.A.) for transfection and expression in mammalian cell lines. The human ASGP-2 cDNA and HC11 cells transfected with human ASGP-2 construct were generously provided by Dr Aymee Perez and Jin Zhang (University of Miami, Miami, FL, U.S.A.), respectively. Cos-7 and HBL-100 cells were grown in Dulbecco's modified Eagle's medium (DMEM) low-glucose media and DMEM high-glucose media (Gibco-BRL), respectively, supplemented with 10% fetal calf serum, penicillin (100 i.u./ml) and streptomycin (100 mg/ml). MCF-7 cells were grown in DMEM high-glucose media supplemented with 10% fetal calf serum, insulin (5 µg/ml; Sigma), non-essential amino acids (1 mM; Gibco-BRL), penicillin (100 i.u./ml) and streptomycin (100 mg/ml). Each of these cell lines was transfected with cDNAs for Muc4/SMC analogues

or ASGP-2 [8] using LipofectAMINE Reagent (Life Technologies). Cells were cultured in G418 (0.5 mg/ml) containing media for selection of stable transfectants. HC11 mouse mammary epithelial cells were grown in complete medium: RPMI 1640 (Gibco-BRL), L-glutamine, 50 µg/ml gentamicin, 10% fetal calf serum, 10 ng/ml EGF (Sigma) and 5 µg/ml insulin.

Human ASGP-2 cDNA was cloned into the pCDNA3/myc-His vector (Invitrogen) with C-terminal *c-myc* and 6×His epitopes in frame with the cathepsin D signal sequence. HC11 and Cos-7 cells were transiently transfected with the human ASGP-2 construct (J. Zhang and K. L. Carraway, unpublished work) using the LipofectAMINE Plus Reagent according to manufacturer's protocol (Gibco Life Technologies). For transient transfectants, cells were harvested or used for assays 2–3 days after the transfection. To obtain conditioned media, transfectants were cultured for 3 days in serum-free media supplemented with insulin, transferrin and sodium selenite (ITS; Gibco-BRL), starting 24 h after transfection. Deletion constructs were made by PCR using primers specific to the sequence between EGF-2 and transmembrane domains with an overhang of an *AatII* or *BglII* restriction site.

Immunoblotting

SDS/PAGE was performed under reducing conditions using 8% or 14% polyacrylamide gels and the mini-Protein II system (Bio-Rad). Resolved proteins were transferred to nitrocellulose filters, which were subsequently blocked with 5% non-fat dry milk in TTBS buffer (Tris-buffered saline/0.5% Tween 20). The filters were incubated in primary antibody diluted in 1% BSA/TTBS for 1 h, then in horseradish peroxidase-conjugated goat anti-mouse IgG (Promega) diluted 1:14000 for 1 h. The signal was detected with a Renaissance Enhanced Chemiluminescence Kit (DuPont NEN).

Immunoprecipitations

Immunoprecipitation was performed with 30 µl of Protein A-agarose (Sigma) and 15 µl of antiserum. Immunoprecipitation tubes were rotated overnight at 4 °C and the resulting precipitates were washed five times in the appropriate buffer. Bound proteins were released by boiling in SDS sample buffer and analysed by Western blotting.

Pulse-chase studies of Muc4/SMC synthesis

Transfectants were grown subconfluently in 60 mm tissue-culture dishes. Cells were rinsed three times with PBS and incubated in prelabelling medium (Met/Cys-free DMEM supplemented with 10% dialysed fetal calf serum) for 30 min at 37 °C. Cells were then metabolically labelled with 100 µCi of [³⁵S]Met/Cys (NEN Life Science Products) in 0.6 ml of prelabelling media at 37 °C. After a 10 min pulse labelling, cells were washed twice with PBS and incubated in the growth media for the chase period. At each time point, cells were lysed in 0.5 ml of SDS lysis buffer (0.5% SDS, 5 mM Tris and 5 mM EDTA, pH 8.3), boiled for 5 min, and sonicated. To the SDS lysate, 0.1 ml of 1 M Tris, pH 8.0, 170 µl of 10% Triton X-100 and protease inhibitor cocktail (Boehringer Mannheim) were added. After a 1 h incubation at room temperature, the cell lysate was centrifuged at 14000 g for 15 min. The cleared lysate was immunoprecipitated with anti-ASGP-2 or anti-C-pep polyclonal antibodies. Immunoprecipitates were analysed by SDS/PAGE and fluorography.

Isolation of C-pep fragment by immunoaffinity column

An anti-C-pep affinity column was prepared using anti-C-pep polyclonal antibodies [15] and Immunopure Protein A IgG Orientation Kit (Pierce). Colon tissue dissected from female Fischer 344 rats was pulverized with a mortar and pestle in liquid N₂ and stored as a powder at -80 °C. Tissue powder was solubilized by boiling in SDS lysis buffer for 10 min and homogenized with a probe sonicator. The tissue lysate was then treated with Triton X-100 and protease inhibitors and centrifuged at 14000 *g* as described above. The cleared lysate was diluted 1:1 with column binding buffer (10 mM Tris buffer, pH 7.5) and applied to the immunoaffinity column. After washing the column with binding buffer, membrane-bound ASGP-2 and the cleaved product (C-pep fragment) were eluted with elution buffer (0.1 M glycine/HCl buffer, pH 2.8). The pH of each eluted fraction was raised to neutral with 1 M Tris/HCl, pH 9.5. Fractions 3, 4 and 5 (1 ml/fraction) were combined and concentrated in a Centricon 3 (Amicon). The concentrated eluate was analysed by SDS/PAGE followed by Western blotting.

RESULTS

Muc4/SMC is secreted by proteolytic cleavage of membrane precursor

Previously, we described the presence of the soluble form of Muc4/SMC in several normal rat glandular epithelial tissues [15,23,25]. The soluble form was also detected in human milk [15], tears [19] and saliva [17]. Soluble Muc4/SMC is a truncated form that lacks transmembrane and cytoplasmic domains [5,15]. Since no alternative splicing has been observed [15], we tested for a proteolytic mechanism for releasing the extracellular portion of the Muc4/SMC molecule. Definitive evidence for a proteolytic mechanism for producing soluble Muc4/SMC would be provided by demonstrating that the truncated soluble form can be produced from the cDNA encoding the membrane-bound form of Muc4/SMC. Thus cDNA for the integral membrane Muc4/SMC

Figure 1 Soluble Muc4/SMC produced by transfected human tumour cell lines

Cell lines expressing transfected Muc4/SMC were grown for 3 days in culture. Medium was removed from each culture for SDS/PAGE and immunoblot analyses. Cells were then lysed and lysates prepared for SDS/PAGE and immunoblotting. Immunoblots were performed with anti-ASGP-2 mAb 13C4, which recognizes both the soluble and membrane forms of Muc4/SMC, and anti-ASGP-2 polyclonal antibody C-pep, which recognizes only the membrane form.

Figure 2 Soluble Muc4/SMC produced by transfected mouse epithelial cell line

HC11 cells expressing transfected Muc4/SMC were lysed for immunoprecipitation (IP) with anti-C-pep. After three sequential immunoprecipitations with anti-C-pep to completely remove the membrane form, the soluble form was immunoprecipitated from the resulting supernatant with anti-ASGP-2 polyclonal antibody. All immunoprecipitates were immunoblotted with anti-ASGP-2 mAb 4F12. Control (Cont.) lanes contain immunoprecipitates with non-immune antisera: control 1 for the anti-C-pep immunoprecipitation and control 2 for the anti-ASGP-2 immunoprecipitation.

was transfected into various mammalian cell lines. Following transfection, cells were cultured in serum-free media supplemented with ITS. Conditioned media from these transfectants were harvested, filtered through 0.22 µm cellulose acetate filters and centrifuged at 100000 *g* for 1 h. The supernatant was concentrated 10–30-fold using a Centricon 50 (Amicon). The conditioned media and whole cell lysates were then analysed for the presence of Muc4/SMC protein by SDS/PAGE followed by immunoblotting with anti-Muc4/SMC monoclonal antibody (mAb) 13C4 specific to ASGP-2 [15]. In all cell lines tested [human melanoma A375, breast cancer MCF-7, simian virus 40-transformed monkey kidney fibroblast Cos-7 (not shown) and simian virus 40-transformed human HBL-100 cells], ASGP-2 was readily detectable by 13C4 mAb in both cell lysates and conditioned media (Figure 1). The same samples were also blotted with anti-C-pep antibodies specific to the cytoplasmic tail of Muc4. These analyses revealed that Muc4/SMC found in the media lacks the C-terminal sequence whereas the Muc4/SMC in the cell still retains the C-terminal sequence, demonstrating the production of the two forms from a single message (Figure 1). Since cDNAs used for transfection were generated from mRNA of integral membrane Muc4/SMC, alternative splicing is ruled out as a possible mechanism for Muc4/SMC secretion in these transfectants. Therefore, these data provide the first evidence for synthesis of the soluble form via post-translational proteolytic cleavage of the membrane-bound Muc4. Although missing the cytoplasmic domain, the 13C4-reactive Muc4/SMC in the conditioned media appears otherwise intact. This result suggests that the cleavage is mediated by a specific endogenous proteolytic enzyme in the cells. Further, the secretion from melanoma A375 cells and fibroblastic Cos-7 cells (not shown) also suggests that this secretion mechanism is not restricted to the secretory epithelial cells but may be universal.

Production of soluble Muc4/SMC in normal mammary epithelial cells

The analyses of the secretion mechanism described above were performed using transformed/neoplastic cell lines. It is possible, however, that the observed secretion of Muc4/SMC by proteolytic cleavage in these cells represents an aberrant secretion mechanism used by neoplastic cells. To examine whether the

Figure 3 Secretion of human MUC4 by transfected cell lines

HC11 and Cos-7 cell lines were transfected with a construct for human MUC4. Cell lysates were analysed for soluble MUC4 by sequential immunoprecipitation (IP) with anti-C-pep and anti-ASGP-2 as described previously [15]. Control lanes are as in Figure 2.

soluble form of Muc4/SMC can be produced via post-translational proteolysis in normal epithelial cells, Muc4/SMC cDNA was transfected into the HC11 mouse mammary epithelial cells. The presence of soluble Muc4/SMC was determined in the lysate of transfected cells by a serial immunoprecipitation procedure (Figure 2). After the depletion of membrane-bound Muc4/SMC by three rounds of immunoprecipitation with anti-C-pep antibody, which recognizes the cytoplasmic domain of Muc4/SMC

[15], the remaining Muc4/SMC (soluble form) was precipitated using polyclonal anti-ASGP-2 antibody. Immunoprecipitates from each step were blotted with 4F12 anti-ASGP-2 mAb. These analyses demonstrated that non-transformed epithelial cells also produce the soluble form from the membrane precursor by a proteolytic mechanism. These observations in HC11 cells suggest that the proteolytic mechanism is a physiological mechanism for the production of soluble Muc4.

Human MUC4 can also be secreted by a proteolytic mechanism

Human MUC4 is also present as membrane-bound and soluble forms. The secretion of MUC4 has been detected in human lacrimal gland [16] and salivary gland [20], as well as in tears [26] and milk (J. Zhang and K. L. Carraway, unpublished work). Given the high homology of the amino acid sequences of MUC4 and Muc4 between human and rat, the soluble form of human MUC4 may be produced by a similar proteolytic mechanism to that observed for rat Muc4/SMC. To determine the secretion mechanism of human MUC4, we transfected the cDNA for the transmembrane subunit ASGP-2 of the human MUC4 into HC11 and Cos-7 cells and examined the production of the soluble form from the membrane-bound MUC4. The serial immunoprecipitation experiments demonstrated that a soluble form of MUC4 was produced in both epithelial (HC11) and fibroblastic (Cos-7) cells (Figure 3). These observations argue that human MUC4 can also be secreted by the proteolytic mechanism and that it possesses a specific site for cleavage to release the extracellular portion of the molecule from the cells.

Figure 4 Analysis of membrane-associated fragment from proteolytic cleavage and secretion of Muc4/SMC

A375 cells with tetracycline-regulated Muc4/SMC turned On and Off, MCF-7 cells expressing or not expressing Muc4/SMC and rat salivary gland [17] were lysed and prepared for SDS/PAGE and immunoblotting with anti-C-pep. Lysates of rat colon [15], lacrimal gland [16] and mammary gland [15] were fractionated by affinity chromatography on anti-C-pep and the bound fraction prepared for SDS/PAGE and immunoblotting with anti-C-pep. Arrows show the positions of bands for intact ASGP-2 (top) and the membrane-associated fragment (bottom).

Figure 5 Analysis of potential cleavage region using Muc4/SMC deletion mutants

(A) Deletion constructs and effects on secretion. (B) Immunoblot analysis of secretion of EGF2 deletion mutant. cDNA for the SMC deletion mutant lacking EGF2 and the rest of the extracellular sequence was transiently transfected into HBL-100 cells. Production of soluble isoform was examined by Western blot analysis of cell lysate and the conditioned media using anti-ASGP-2 mAb 4F12. (C) Sequential immunoprecipitation analysis of synthesis of soluble form using the EGF(1 + 2) deletion mutant. The cDNA construct for the deletion mutant was transiently transfected into HBL-100 cells. Production of soluble isoform was examined by sequential immunoprecipitation with anti-C-pep and anti-ASGP2 polyclonal antibodies followed by Western blotting with anti-ASGP2 mAb 4F12 as described in Figure 2.

That the mechanism is present in human cells is evident from the studies on the three human cell lines (Figure 1).

Membrane-bound Muc4/SMC is released from the cell by cleavage between the two EGF-like domains

In the analyses using transfected cell lines, the bands of the ASGP-2 subunit of the membrane-bound form and the soluble form seem to be discrete and roughly identical in size by SDS/PAGE (Figures 1–3), suggesting that the Muc4/SMC molecule is cleaved at a specific site in the extracellular domain close to the plasma membrane. Since Muc4/SMC has a short cytoplasmic tail of only 20 amino acids, such a proteolytic cleavage should yield a small fragment of the C-terminal peptide composed of the cytoplasmic tail, transmembrane domain and a small portion of the extracellular domain. To facilitate the size

comparison, ASGP-2 subunit of the two forms was deglycosylated using a combination of glycosidases. Supporting the idea of cleavage at the peri-membrane region, the size comparison indicated that the soluble form is approx. 20–25 kDa smaller than the membrane form (results not shown). In an effort to identify the proteolytic cleavage site, we next determined the size of C-terminal peptide fragment. SDS lysates were prepared from various cell types and tissues, separated by SDS/PAGE (14% gel), and immunoblotted with anti-C-pep antibodies (Figure 4). These analyses demonstrated the size of the C-peptide fragment to be approx. 25 kDa (Figure 4). Importantly, these fragments were apparently identical in size across the different cells and tissues, suggesting an identical mechanism and the use of a common proteolytic enzyme at the specific site in all the cells tested, including those from mammary, colon, trachea, lacrimal gland and salivary gland. Thus the identification of the C-peptide

Figure 6 Pulse-chase analysis of Muc4/SMC synthesis and cleavage

Pulse-chase studies were performed as described in the Experimental section. IP, immunoprecipitation.

fragment in tissues provided further compelling evidence that the proteolytic cleavage of the membrane-bound Muc4/SMC is not unique in the transfection models, and that it is a mechanism utilized for Muc4/SMC secretion by normal tissues.

In normal rat tissues tested in Figure 4, multiple bands were observed in the anti-C-pep immunoblot. These bands are most likely the products of other proteolytic processes of Muc4 in these tissues. The expression levels of Muc4 are much lower in normal tissues compared with those in the transfected cell lines. In addition, the 25 kDa fragment appears to degraded in these tissues and disappears quickly after its production. Therefore, the enrichment of C-pep-positive Muc4 using anti-C-pep column was necessary to detect the small amount of 25 kDa fragment. This procedure, of course, enriched all other C-pep-positive Muc4 fragments present, which may have physiological functions in these tissues. For instance, based on the molecular mass, these fragments are expected to retain EGF domains and may function as intramembrane HER2/ErbB2 modulators as demonstrated *in vitro* for the full-length Muc4 [7].

With regard to Muc4 secretion mechanism, the size of the C-terminal peptide fragment suggests that the proteolytic cleavage site resides in the amino acid sequence between the two EGF-like domains of ASGP-2, most likely N-terminal to the last putative N-glycosylation site. Based on the sequence data [6], the C-terminal peptide fragment could contain one N-linked oligosaccharide, adding 3–5 kDa to its molecular mass, which could explain the presence of a smaller/non-glycosylated fragment (approx. 20 kDa) found in colon. To confirm that the region flanked by the two EGF-like domains contains the cleavage site, we produced cDNA constructs with a deletion of this region (Figure 5A) and expressed them in HBL-100 cells to examine the secretion of these mutant Muc4/SMC molecules. The deletion of the amino acid sequence N-terminal to the transmembrane domain, including the EGF-2 domain, did not interfere with Muc4/SMC secretion (Figure 5B). In comparison, soluble Muc4/SMC was not produced after deletion of the sequence between EGF-1 and the transmembrane domain, suggesting that this deletion completely abolished Muc4/SMC secretion by removing the specific proteolytic site (Figure 5C). These studies

further confirm the Muc4/SMC secretion mechanism and the site of proteolytic cleavage.

Release of the extracellular portion of Muc4/SMC takes place at an early stage of Muc4/SMC synthesis

Many membrane-bound cell-surface molecules are released from the cell surface by a shedding mechanism, a proteolytic process involving cell-surface or extracellular proteases [27]. On the other hand, other membrane-bound molecules such as growth factor/ligand precursors are processed to soluble forms within the cell and released to the extracellular environment by exocytosis [28]. To determine which proteolytic mechanism is used for the secretion of Muc4, we studied the biosynthesis of Muc4/SMC in the transfected cells by pulse-chase labelling analyses. HBL-100 cells were transfected and metabolically labelled with [³⁵S]-methionine and [³⁵S]-cysteine for 10 min. The pulse-labelled proteins were chased for up to 4 h. At the indicated time points, cells were lysed in 0.5 % SDS lysis buffer and boiled for 10 min. Triton X-100 was then added to the cell lysate for immunoprecipitation using anti-ASGP-2 or anti-C-pep polyclonal antibodies. Immunoprecipitates were analysed by SDS/PAGE and fluorography (Figure 6). The results provide two important observations. First, the time course for the disappearance of the precursor is essentially the same with both antibodies, indicating that cleavage to remove the cytoplasmic domain does not precede cleavage to yield the two subunits. Secondly, only a small fraction of mature ASGP-2 is precipitated by anti-C-pep, indicating that there is little membrane-associated mature ASGP-2 and that cleavage to remove the cytoplasmic domain does not significantly lag the cleavage to yield the two subunits. These results also indicate that the primary synthetic product in these cells is the soluble form, and that the final ratio of soluble to membrane form (Figure 1) must result from accumulation of the membrane form in the cells. Our current results, therefore, demonstrate that the proteolytic cleavage responsible for the secretion also occurs at an early stage of the biosynthesis pathway, at approximately the same time as cleavage of the precursor Muc4/

SMC into two subunits. In total, these observations suggest that Muc4/SMC is secreted by the cells via intracellular proteolysis followed by active exocytosis. This result is consistent with our previous observation of secretagogue-induced secretion from the colon [15].

In the pulse-chase analyses, it is also noteworthy that ASGP-2 increases its size between 1 and 2 h of chase, resulting in a more heterogeneous broad band of ASGP-2. The heterogeneity of ASGP-2 indicates that this molecule undergoes further post-translational modification over the longer period of time. As expected, treatment of ASGP-2 with the N-glycosidases demonstrated that this increase in molecular mass is due to additional glycosylation of the ASGP-2 subunit (results not shown).

DISCUSSION

In this work we investigated the secretion mechanism of the multifunctional glycoprotein Muc4/SMC. Production of the soluble form following the transfection and expression of a membrane-bound Muc4/SMC demonstrated that soluble Muc4/SMC can be produced and secreted from cells via proteolytic cleavage of membrane precursor. Using a human MUC4 cDNA sequence, we demonstrated that this mechanism is also relevant for human MUC4 secretion. The subsequent analyses on tissue Muc4/SMC demonstrated that the proteolytic mechanism accounts for the production of soluble form in various tissues and glands. The pulse-chase analyses demonstrated that this proteolysis is kinetically indistinguishable from cleavage to produce the two subunits, as shown in the model in Figure 7, providing the first clear evidence for a secretion rather than a shedding mechanism for a membrane mucin. It has been previously determined that the cleavage to yield the two subunits takes place before addition of O-linked oligosaccharides, at about the time of the transition of the precursor Muc4/SMC from endoplasmic reticulum to Golgi [5]. These combined observations suggest an involvement of the activity of a specific endoplasmic reticulum or early Golgi protease in the secretion of Muc4.

We propose that the regulation of this proteolytic activity is of considerable importance for the function of this glycoprotein. In

the malignant rat tumour ascites cells, Muc4/SMC is expressed predominantly in a membrane-bound form, possibly due to the loss of activity or down-regulation of gene expression of the protease in these tumour cells. As a result, the overexpressed membrane mucin anchored to the plasma membrane causes steric hindrance to adhesion molecules, suppresses tumour cell adhesion and promotes tumour dissemination [8,11]. In addition, Muc4/SMC provides an effective barrier to the cell surface, enabling the tumour cells to escape from immune surveillance [29]. The membrane-bound Muc4/SMC also provides an intramembrane mechanism for the activation of receptor tyrosine kinase HER2/ErbB2 [7]. Muc4/SMC expressed in A375 melanoma cells exhibits the sialyl Lewis^x carbohydrate epitope, a ligand for P- and E-selectin, on the mucin subunit ASGP-1 (M. Komatsu and K. L. Carraway, unpublished work), which may facilitate tumour cell homing to the target organs during the metastatic process [11]. All of these findings from the functional studies and the lack of Muc4/SMC secretion in these malignant cells suggest that some neoplastic cells may acquire increased metastatic potential upon down-regulation of the proteolysis of membrane-bound Muc4. Therefore, while the importance of up-regulation of Muc4/SMC expression has been emphasized for the progression of cancer, it should also be considered that the down-regulation of proteolytic cleavage could be an equally critical step leading to the increased malignancy.

The membrane-bound precursor is cleaved within the region flanked by the two EGF-like domains. Thus this cleavage releases Muc4/SMC containing the EGF-1 domain and leaves the EGF-2 domain anchored to the cell surface. It is not known whether the EGF-1 domain of the secreted Muc4/SMC can act as a stimulatory ligand for ErbB2, as demonstrated for membrane-bound Muc4/SMC [7]. It is, however, interesting to note that Muc4/SMC is abundantly secreted into milk in rats [15] and humans (J. Zhang and K. L. Carraway, unpublished work). Another member of the membrane mucin family MUC3 also contains two EGF-like domains close to the plasma membrane [30]. MUC3 can be expressed in soluble form by alternative splicing [31]. Interestingly, this splice variant of MUC3 contains only the first of the two EGF-like domains. It is not known whether the EGF-like domains of MUC3 exhibit any functional activity.

Most normal tissues expressing Muc4/SMC produce both membrane and soluble forms. However, the relative abundance of each form depends on the tissue type [23], suggesting the differential physiological role of each form in each tissue. Soluble Muc4/SMC is secreted into fluids such as saliva, tear fluid and milk, and may serve protective, lubricating and/or anti-microbial roles, as demonstrated for other mucins [32]. Following secretion into the extracellular environment, soluble Muc4/SMC can also associate with the cell and provide a mucus layer at the apical surface. For instance, 40% of rat uterine Muc4, corresponding to the soluble fraction, is removed by rinsing uterine preparations with saline, an indication that the soluble form is adsorbed loosely to the uterine lining [14].

In summary, these observations provide strong evidence that membrane mucins can be secreted from cells rather than released from cell surfaces by a shedding mechanism. This secretion provides an additional mechanism for regulating the release of mucins into physiological fluids, such as tears, in response to physiological stimuli and secretagogues involved in the protection of epithelial surfaces.

Proteolytic cleavage and production of soluble SMC

Figure 7 Model for the synthesis of membrane (1) and soluble (2) forms of Muc4/SMC

This research was supported in part by grant CA52498 from the National Institutes of Health and by the Sylvester Comprehensive Cancer Center of the University of Miami. We thank Dr Aymee Perez for providing the construct for human MUC4 and

Jin Zhang for providing the transfected HC11 cells and for help in preparing the manuscript. We also thank Jian Zhang for assistance with the immunoaffinity chromatography.

REFERENCES

- Sherblom, A. P. and Carraway, K. L. (1980) A complex of two cell surface glycoproteins from ascites mammary adenocarcinoma cells. *J. Biol. Chem.* **255**, 12051–12059
- Porchet, N., Nguyen, V. C., Dufosse, J., Audie, J. P., Guyonnet Duperat, V., Gross, M. S., Denis, C., Degand, P., Bernheim, A. and Aubert, J. P. (1991) Molecular cloning and chromosomal localization of a novel human tracheo-bronchial mucin cDNA containing tandemly repeated sequences of 48 base pairs. *Biochem. Biophys. Res. Commun.* **175**, 414–422
- Moniaux, N., Nollet, S., Porchet, N., Degand, P., Laine, A. and Aubert, J.-P. (1999) Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin. *Biochem. J.* **328**, 325–333
- Sheng, Z., Hull, S. R. and Carraway, K. L. (1990) Biosynthesis of the cell surface sialomucin complex of ascites 13762 rat mammary adenocarcinoma cells from a high *M_r* precursor. *J. Biol. Chem.* **265**, 8505–8510
- Carraway, K. L., Fregien, N., Carraway, C. A. C. and Carraway, III, K. L. (1992) Tumor sialomucin complexes as tumor antigens and modulators of cellular interactions and proliferation. *J. Cell Sci.* **103**, 299–307
- 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 family superfamily. *J. Biol. Chem.* **267**, 16341–16346
- Carraway, III, K. L., Rossi, E. A., Komatsu, M., Price-Schiavi, S. A., Huang, D., Carvajal, M. E., Guy, P. M., Fregien, N., Carraway, C. A. C. and Carraway, K. L. (1999) An intramembrane ErbB2/Neu ligand that potentiates neuregulin signaling. *J. Biol. Chem.* **274**, 5263–5266
- Komatsu, M., Carraway, C. A. C., Fregien, N. L. and Carraway, K. L. (1997) Reversible disruption of cell-matrix and cell-cell interactions by overexpression of sialomucin complex. *J. Biol. Chem.* **272**, 33245–33254
- Komatsu, M., Yee, L. and Carraway, K. L. (1999) Overexpression of sialomucin complex, a rat homolog of MUC4, inhibits tumor killing by lymphokine-activated killer cells. *Cancer Res.* **59**, 2229–2236
- Komatsu, M., Jepson, S., Arango, M. E., Carraway, C. A. C. and Carraway, K. L. (2001) MUC4/sialomucin complex, an intramembrane regulator of ErbB2/HER2/Neu, suppresses apoptosis and potentiates primary tumor growth in a xenotransplanted tumor. *Oncogene* **20**, 461–470
- Komatsu, M., Tatum, L., Altman, N. H., Carraway, C. A. C. and Carraway, K. L. (2000) Potentiation of metastasis by cell surface sialomucin complex (rat MUC4), a multifunctional anti-adhesive glycoprotein. *Int. J. Cancer* **87**, 480–486
- Jepson, S., Komatsu, M., Haq, B., Arango, M. E., Huang, D., Carraway, C. A. C. and Carraway, K. L. (2002) Muc4/sialomucin complex, the intramembrane ErbB2 ligand, induces specific phosphorylation of ErbB2 and enhances expression of p27^{kip}, but does not activate mitogen-activated kinase or protein kinase B/Akt pathways. *Oncogene* **21**, 7524–7532
- 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
- Idris, N. and Carraway, K. L. (1999) Sialomucin complex (Muc4) expression in the rat female reproductive tract. *Biol. Reprod.* **61**, 1431–1438
- Rossi, E. A., McNeer, R., Price-Schiavi, S. A., Komatsu, M., Van den Brande, J. M. H., Thompson, J. F., Carraway, C. A. C., Fregien, N. L. and Carraway, K. L. (1996) Sialomucin complex, a heterodimeric glycoprotein complex: expression as a soluble, secreted form in lactating mammary gland and colon. *J. Biol. Chem.* **271**, 33476–33485
- Arango, M. E., Li, P., Komatsu, M., Montes, C., Carraway, C. A. C. and Carraway, K. L. (2001) Production and localization of Muc4/sialomucin complex and its receptor tyrosine kinase ErbB2 in the rat lacrimal gland. *Invest. Ophthalmol. Vis. Sci.* **42**, 2749–2756
- Li, P., Arango, M. E., Perez, R. E., Reis, C. A., Bonfante, E. L., Weed, D. and Carraway, K. L. (2001) Expression and localization of immunoreactive-sialomucin complex (Muc4) in salivary glands. *Tissue Cell* **33**, 111–118
- Price-Schiavi, S. A., Carraway, C. A. C., Fregien, N. and Carraway, K. L. (1998) Post-transcriptional regulation of a milk membrane protein, the sialomucin complex [ascites sialoglycoprotein (ASGP)-1/ASGP-2, rat Muc4] by TGF β . *J. Biol. Chem.* **273**, 35228–35237
- Price-Schiavi, S. A., Meller, D., Jing, X., Merritt, J., Carvajal, M. E., Tseng, S. C. G. and Carraway, K. L. (1998) Sialomucin complex at the rat ocular surface: a new model for ocular surface protection. *Biochem. J.* **335**, 457–463
- Weed, D. T., Gomez-Fernandez, C., Bonfante, E., Lee, T. D., Pacheco, J., Carvajal, M. E., Goodwin, W. J. and Carraway, K. L. (2001) MUC4 (sialomucin complex) expression in salivary tumors and squamous cell carcinoma of the upper aerodigestive tract. *Otolaryngol. Head Neck Surg.* **124**, 127–141
- Carraway, K. L. (2000) Preparation of membrane mucin. In *Methods in Molecular Biology*, vol. 125, Glycoprotein Methods and Protocols: The Mucins (Corfield, T., ed.), pp. 15–26, Humana Press, Totowa, NJ
- Gendler, S. J. and Spicer, A. P. (1995) Epithelial mucin genes. *Annu. Rev. Physiol.* **57**, 607–634
- Carraway, K. L., Perez, A., Idris, N., Jepson, S., Arango, M., Komatsu, M., Haq, B., Price-Schiavi, S. A., Zhang, J. and Carraway, C. A. C. (2001) Muc4/sialomucin complex, the intramembrane ErbB2 ligand, in cancer and epithelia: to protect and to survive. *Progr. Nucleic Acids Res. Mol. Biol.* **171**, 149–185
- Moniaux, N., Escande, F., Batra, S. K., Porchet, N., Laine, A. and Aubert, J.-P. (2000) Alternative splicing generates a family of putative secreted and membrane-associated MUC4 mucins. *Eur. J. Biochem.* **267**, 4536–4544
- McNeer, R. R., Price-Schiavi, S., Komatsu, M., Fregien, N., Carraway, C. A. C. and Carraway, K. L. (1997) Sialomucin complex in tumors and tissues. *Front. Biosci.* **2**, 449–459
- Pflugfelder, S. C., Liu, Z., Monroy, D., Li, D.-Q., Carvajal, M. E., Price-Schiavi, S. A., Idris, N., Solomon, A., Perez, A. and Carraway, K. L. (2000) Detection of sialomucin complex (MUC4) in human ocular surface epithelium and tear fluid. *Invest. Ophthalmol. Vis. Sci.* **41**, 1316–1326
- Rose-John, S. and Heinrich, P. C. (1994) Soluble receptors for cytokines and growth factors: generation and biological function. *Biochem. J.* **300**, 281–290
- Burgess, T. L. and Kelly, R. B. (1987) Constitutive and regulated secretion of proteins. *Annu. Rev. Cell Biol.* **3**, 243–293
- Moriarty, J., Skelly, C. M., Bharathan, S., Moody, C. E. and Sherblom, A. P. (1990) Sialomucin and lytic susceptibility of rat mammary tumor ascites cells. *Cancer Res.* **50**, 6800–6805
- Williams, S. J., Munster, D. J., Quin, R. J., Gotley, D. C. and McGuckin, M. A. (1999) The MUC3 gene encodes a transmembrane mucin and is alternatively spliced. *Biochem. Biophys. Res. Commun.* **261**, 83–89
- Crawley, S. C., Gum, Jr, J. R., Hicks, J. W., Pratt, W. S., Aubert, J. P., Swallow, D. M. and Kim, Y. S. (1999) Genomic organization and structure of the 3' region of human MUC3: alternative splicing predicts membrane-bound and soluble forms of the mucin. *Biochem. Biophys. Res. Commun.* **263**, 728–736
- Strous, G. and Dekker, J. (1992) Mucin-type glycoproteins. *Crit. Rev. Biochem. Mol. Biol.* **27**, 57–92

Received 4 June 2002/18 July 2002; accepted 20 August 2002

Published as BJ Immediate Publication 20 August 2002, DOI 10.1042/BJ20020862