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Enzymatic Cleavage as a Processing Step in the Maturation of Muc4/Sialomucin Complex

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Abstract Cleavage of Muc4/SMC precursor into two subunits is an essential processing step for maturation and occurs within a GD-PH sequence. Recent evidence indicates that cleavage of the precursor of gel-forming mucin MUC2 within the same tetrapeptide sequence occurs by a non-enzymatic, autocatalytic cleavage at low pH, and in cells in the late secretory pathway. Here we provide evidence that the cleavage step of Muc4/SMC processing occurs by a proteolytic mechanism. First, processing of Muc4/SMC precursor to ASGP-2 was inhibited in the presence of the mechanism-based serine protease inhibitor, Pefabloc SC, under conditions that did not block synthesis of other proteins. This inhibition led to an increased level of the precursor. Second, neutralization of the acidic environment of the late secretory pathway with NH_4Cl did not inhibit cleavage of Muc4/SMC precursor. These results indicate that the two mucins can be processed by cleavage at the same peptide site by different mechanisms. J. Cell. Biochem. 97: 1267–1274, 2006. © 2005 Wiley-Liss, Inc.

Key words: Muc4; biosynthesis; proteolysis; precursor

Muc4/sialomucin complex (SMC), the rat homologue of human MUC4, is a membrane bound heterodimeric glycoprotein complex derived from a single gene that is post-translationally processed into two subunits [Sheng et al., 1990]. The first, a mucin subunit, ascites sialoglycoprotein-1 (ASGP-1) is a high molecular weight sialomucin that is highly *O*-glycosylated. The second, a transmembrane subunit (ASGP-2) that anchors the complex to the plasma membrane, is *N*-glycosylated and contains two epidermal growth factor domains (EGF-1 and EGF-2) [Sheng et al., 1992]. Muc4/SMC was originally isolated from highly malignant ascites 13762 rat mammary adenocarcinoma cells [Sherblom and Carraway, 1980; Sherblom et al., 1980]. Muc4/SMC is multifunctional, providing cells with anti-adhesive [Komatsu et al., 1997], anti-recognition [Komatsu et al., 1999], and signal regulatory functions [Carraway et al., 1999, 2003], all of which are properties that are important to both normal epithelia and tumors. Its anti-adhesive property functions to blocks cell–cell and cell–matrix interactions and to promote metastasis. Its anti-recognition property is largely attributed to the steric hindrance of the rigid and extended high molecular weight mucin subunit, and enables cells to escape immune surveillance [Komatsu et al., 1999]. Its signal regulatory property is due to its ability to form multimeric complexes with other signaling molecules, primarily ErbB2 [Carraway et al., 1999; Jepson et al., 2002]. In complex with ErbB2, Muc4/SMC is able to modulate tyrosine phosphorylation of the receptor via the Muc4/SMC EGF domain 1. Muc4/SMC expression is found in a number of different vulnerable epithelia, including those of the female reproductive tract, airway, ocular surface, and mammary gland [Price-Schiavi et al., 2000; Carraway et al., 2001a,b]. Muc4/SMC has also been studied in both rat and

Abbreviations used: TGF, transforming growth factor; SMC, sialomucin complex; ASGP, ascites sialoglycoprotein; MEC, primary mammary epithelial cells; FCS, fetal calf serum; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

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human mammary carcinomas, where overexpression appears to be related to tumor aggressiveness [Price-Schiavi et al., 2000].

A critical step in the expression of Muc4/SMC is the cleavage of its precursor to yield the two subunits. Other mucins, including MUC1 and MUC2 are also produced from precursor forms via a cleavage [Sheng et al., 1990; Carraway et al., 2001a; Lidell et al., 2003]. MUC2 is cleaved at the same GDPH tetrapeptide site previously observed for the Muc4/SMC cleavage. Interestingly, recent studies have shown that this site can be cleaved by an autocatalytic mechanism in MUC2 in vitro and that this acid-dependent cleavage occurs similarly in the late stages of the secretory pathway in cells [Lidell et al., 2003]. It was proposed that other proteins containing this sequence, including Muc4/SMC, are cleaved by the same mechanism. However, this explanation for the cellular cleavage of Muc4/SMC is problematic for two reasons. First, we have shown that the cleavage of Muc4/SMC precursor occurs early in the transit to the cell surface [Sheng et al., 1990] in compartments that are less acidic. Second, we have shown that this processing step can be inhibited by transforming growth factor (TGF)-β [Price-Schiavi et al., 2000; Soto et al., 2003] via the Smad pathway in a process that exhibits no apparent alterations of the glycosylation of ASGP-2. Thus, it is unlikely that the TGF-β is affecting the acidity of the organelles processing Muc4/SMC and more likely that it is affecting the expression of a protease or protease inhibitor involved in Muc4/SMC precursor cleavage. To address the mechanism of the Muc4/SMC precursor cleavage, we have investigated the effects of a series of protease inhibitors. One of these, Peflabloc SC, strongly inhibited the processing to yield ASGP-2. Inhibition of processing resulted in an increase of precursor. Furthermore, unlike MUC2, neutralization of the acidic environment in the late secretory pathway with NH_4Cl did not inhibit Muc4/SMC precursor processing [Lidell et al., 2003], but did affect ASGP-2 glycosylation. These results suggest that the cleavage of the mucins MUC2 and Muc4/SMC occur at the same GDPH site, but by different mechanisms.

MATERIALS AND METHODS

Materials

Anti-ASGP-2 polyclonal antiserum was prepared against purified ASGP-2, as previously described [Vanderpuye et al., 1988]. The mouse monoclonal antibody (mAb) 4F12, which was elicited using purified Muc4/SMC, recognizes an epitope in the N-terminal 53 amino acids of ASGP-2 [Rossi et al., 1996]. Cell culture materials were obtained from Life Technologies, Inc. (Rockville, MD). Monoclonal anti-β-actin antibody was purchased from Sigma (Saint Louis, MO). Pefabloc SC was ordered from Roche (Indianapolis, IN).

Cell Culture

A375 human melanoma cells were grown in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The A375 cell lines with tetracycline-responsive inducible expression of Muc4/SMC analogues were generated previously by stable transfection of recombinant cDNAs [Komatsu et al., 1997]. Transfectants were maintained in complete medium containing G418 (0.8 mg/ml), hygromycin (0.3 mg/ml), and tetracycline (2 mg/ml) for maintenance of genes and repression of Muc4/SMC expression.

Protease Inhibition

Broad-based protease inhibitors specific for aspartic (Pepstatin, 1 μM) (Calbiochem), caspase (YVAD-CHO, 100 μM) (Calbiochem), cysteine (E-64, 10 μM; Leupeptin, 100 μM) (Calbiochem), metallo (TAPI-2, 100 μM) (Calbiochem), serine (Pefabloc SC, 100–400 μM; PMSF, 1 mM; Leupeptin, 100 μM) (Roche Diagnostics) proteases and amino peptidases, and exopeptidases (Bestatin, 10 mM) (Calbiochem) were used to study the cleavage reaction. Cell cultures were pre-treated with the individual protease inhibitors for a period of 1 h at 37°C in complete medium. Cells were starved for 1 h and pulsed for 30 min with ^{35}S in Cys/Met-free medium, washed, and chased in complete medium for specified periods of time. Cell lysates were immunoprecipitated with anti-ASGP-2 antibody and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). No inhibitors were added to the control. Fractions of supernatants from immunoprecipitations were analyzed by SDS–PAGE to determine if protein synthesis was inhibited. To study the long-term effects of the inhibitors on Muc4/SMC processing, cell cultures were treated with or without protease inhibitors for a period of 24 h over a range of different concentrations. After the 24-h incubation period, whole cell lysates

were analyzed by SDS–PAGE and immunoblotting.

Neutralizing Secretory Pathway

Transfectant A375-Rep5 cells were pre-incubated with or without 25 mM NH_4Cl for 12 h followed by 1-h starvation and 8-h labeling period with ^{35}S (NH_4Cl in media). Lysates were immunoprecipitated with anti-ASGP-2 pAb and analyzed by SDS–PAGE. No NH_4Cl was added to control cultures. For Western blotting, cultures were treated as above followed by immunoblotting with 4F12 monoclonal anti-ASGP-2 antibody.

RESULTS

Inhibition of Muc4/SMC Processing by Peflabloc SC

Muc4/SMC is post-translationally processed into two subunits [Sheng et al., 1990], a mucin (ASGP-1) and a transmembrane (ASGP-2) subunit after *N*-glycosylation in the endoplasmic reticulum and prior to *O*-glycosylation [Carraway et al., 1992]. To determine if this cleavage is mediated by a protease, we sought to find an inhibitor for the process. A wide range of broad-based inhibitors specific for aspartic, caspase, cysteine, metallo, and serine proteases were used to study the cleavage reaction. A375 human melanoma cells, transfected with Muc4/SMC regulated by a Tet-off system, were pretreated with the individual protease inhibitors for a period of 1 h at 37°C in complete medium. This cell line overexpresses Muc4/SMC and functions as our model for Muc4/SMC precursor processing [Komatsu et al., 2002]. Pulse labeling experiments were performed to assay for cleavage. Cell lysates from labeled control and treated cells were immunoprecipitated with anti-ASGP-2 antibody, and analyzed by SDS–PAGE and fluorography. As shown in Table I, only Pefabloc SC of the protease inhibitors tested in various experiments was able to repress production of the ASGP-2. Pefabloc SC, at the recommended concentration of 100 μM, completely inhibited Muc4/SMC processing, resulting in the absence of the mature form of the ASGP-2 subunit. Pefabloc SC is a mechanism-based serine protease inhibitor using a sulfonyl fluoride as a reactive group for irreversible inhibition. Leupeptin and PMSF, which are also serine protease inhibitors, did not discernibly inhibit Muc4/SMC processing, suggesting that the specificity of the inhibitors may be different. As controls to show that the protease inhibitors were effective on whole cells, we assayed inhibitors against known cellular proteases. YVAD and E-64 were shown to inhibit caspase and cathepsin B, respectively. The results with pepstatin were equivocal, since the enzyme level was too low. PMSF is already known to penetrate cells and TAPI-2 acts primarily on extracellular or cell surface proteases, so they were not assayed. Most importantly, the purpose of these experiments was to find an inhibitor which blocks the Muc4 precursor cleavage and that was accomplished.

TABLE I. Effects of Protease Inhibitors on ASGP-2 Production From Precursor

Inhibitor	Inhibition
Pepstatin	–
YVAD	–
Pefabloc SC	+
E-64	–
TAPI-2	–
PMSF	–

To verify the inhibition by Peflabloc SC, we analyzed the concentration dependence of inhibition of ASGP-2 production over a longer time period using Western blotting of cell lysates. Cell cultures were treated with Pefabloc SC at concentrations ranging from 25 to 400 μM for 24 h. Muc4/SMC protein expression levels were analyzed by Western blotting with anti-ASGP-2 mAb, 4F12. Protein expression was normalized to β-actin. Concentrations as low as 25 μM were effective in inhibiting Muc4/SMC expression (Fig. 1A,B). Concentrations greater than 100 μM did not further inhibit Muc4/SMC processing. It is important to note, however, that Pefabloc SC concentrations in excess of 150 μM inhibited protein synthesis to some extent (not shown). Altogether, these data provide support for the enzymatic action of a protease, probably a serine protease, in the cleavage step of Muc4/SMC precursor processing.

A trivial explanation of these results is that the Peflabloc SC is inhibiting protein synthesis. To investigate the effect of Pefabloc on protein synthesis, we extended the pulse labeling experiments to 12 h to determine the long-term effects of Pefabloc SC on Muc4/SMC precursor processing and analyzed fractions of the IP supernatants to determine if protein synthesis was inhibited. Cell cultures were pre-incubated

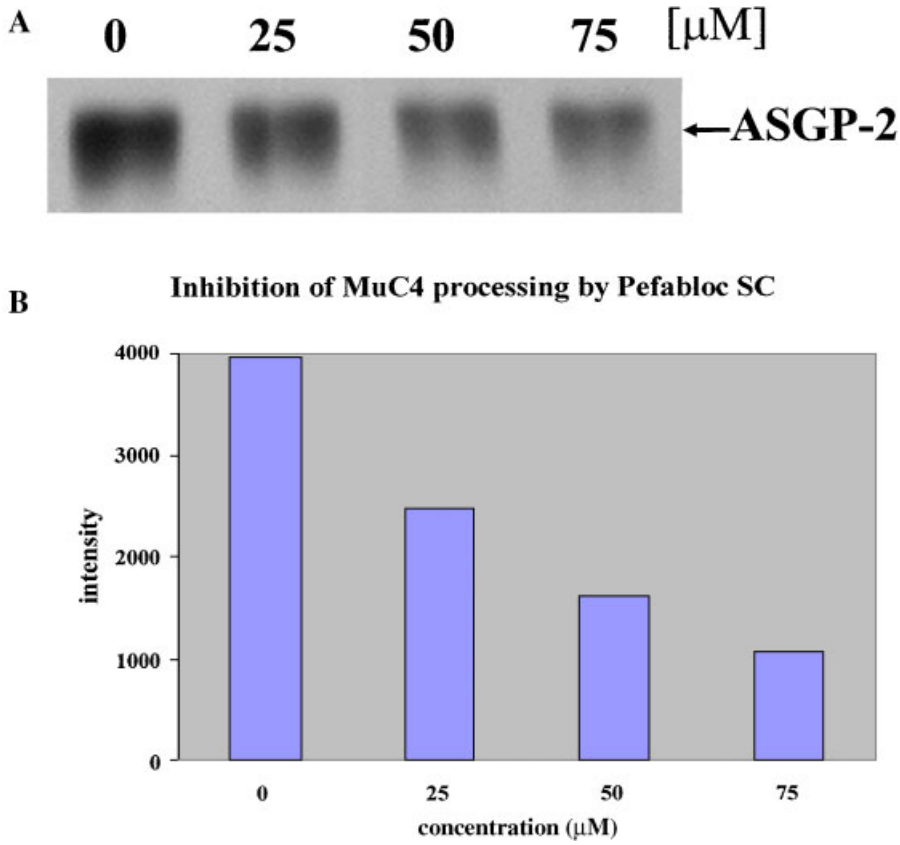

Fig. 1. Concentration dependence of Peflabloc SC inhibition of synthesis of the transmembrane subunit ASGP-2 of Muc4/SMC. **A**: Western blot of transfectant A375-Rep5 cells treated with or without Pefabloc SC (0–75 μM) for a period of 24 h. Lysate was analyzed by immunoblotting with anti-ASGP-2 mAb (4F12). **B**: Graphic representation of A. ASGP-2 levels were normalized to β-Actin.

with the Pefabloc SC (100 μM) for 1 h before adding label. Fluorography of the labeling experiments showed Muc4/SMC processing in controls increasing until the levels of expression begin to diminish sometime between 6 and 12 h (Fig. 2A). Pefabloc SC-treated cultures showed the expected decrease in ASGP-2 levels (Fig. 2B). Fractions from the IP supernatants indicate that protein synthesis was not inhibited in the presence of Pefabloc SC (Fig. 3C,D). The data indicate that Pefabloc SC inhibits Muc4/SMC processing, but has no effect on protein synthesis.

Repression of precursor cleavage should lead to increase in precursor. To address this issue, we examined immunoblots of cells treated with Peflabloc SC at different concentrations. The blots showed an increase in Muc4/SMC precursor with increasing concentrations of Pefabloc SC (Fig. 3). As expected, processed ASGP-2 protein levels decreased with increased Pefabloc SC concentrations. The data indicate that inhibition of Muc4/SMC processing results in accumulation of the precursor form of Muc4/SMC over time.

Neutralization of Secretory Pathway

The protease that cleaves the pMuc4/SMC cleaves at a site (GD-PH) common to MUC2 and MUC5AC, both of which are gel-forming mucins [Carraway et al., 2001a; Lidell et al., 2003]. Cleavage of the precursor form of Muc4/SMC is known to occur at about the time of the transfer from the ER to the Golgi [Sheng et al., 1990]. A cleavage study on MUC2 has suggested an autocatalytic cleavage mechanism that is triggered in the late secretory pathway at low pH (<6.0) [Lidell et al., 2003]. To investigate whether the cleavage of Muc4/SMC that is observed is dependent on the acidic pH levels in the secretory pathway, similar experiments were conducted. The secretory pathway was neutralized by treating cells with NH_4Cl, which mediates its effect by diffusion of

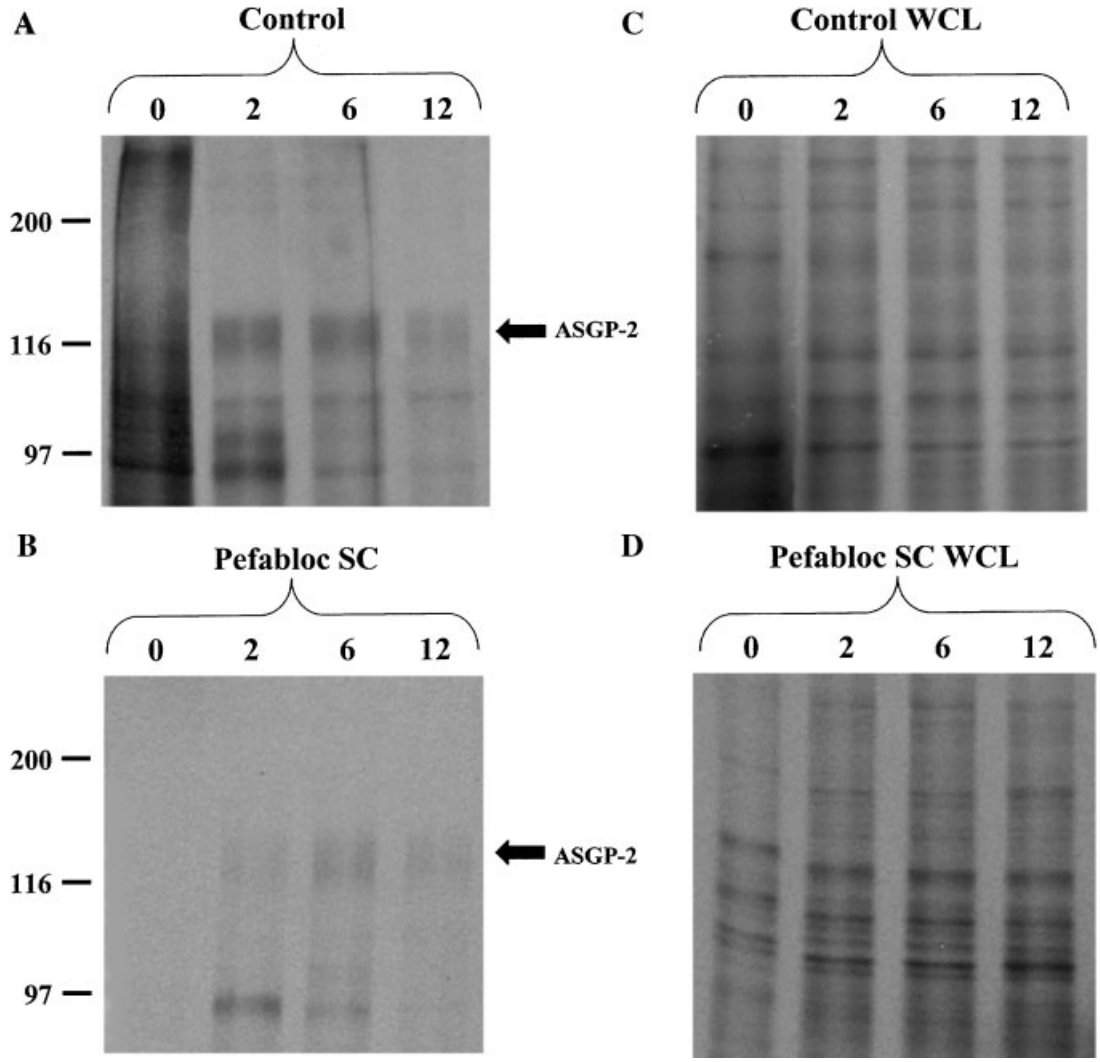

Fig. 2. Peflabloc SC blocks ASGP-2 synthesis without inhibiting protein synthesis. Fluorography of transfectant A375-Rep5 cells that were pre-incubated with or without Pefabloc SC for 1 h. Cells were pulsed for 30 min with 35S, chased and lysed at specified time points. Lysates were immunoprecipitated with anti-ASGP-2 pAb. Immunoprecipitates and non-immunoprecipitated whole cell lysates (WCL) were subjected to SDS–PAGE and fluorography. **A**, non-treated control; **B**, Pefabloc SC-treated (100 μM); **C**, control WCL, and **D**, Pefabloc-treated WCL.

ammonia through the cells [Lidell et al., 2003]. Transfectant A375-Rep5 cells were pre-incubated with or without 25 mM NH_4Cl for 12 h followed by 8-h labeling period with ^{35}S Cys/Met (NH_4Cl in media). Lysates were immunoprecipitated with anti-ASGP-2 pAb and analyzed by SDS–PAGE. Treatment of cells with NH_4Cl did not block cleavage of Muc4/SMC (Fig. 4A). However, in agreement with previous findings on MUC2, we observed a slightly higher mobility of the ASGP-2 subunit in NH_4Cl-treated cells [Axelsson et al., 2001], consistent with previous studies showing that when the secretory pathway is neutralized, with NH_4Cl or bafilomycin A1, a redistribution of glycosyltransferases and decrease in glycan chain length occurs [Axelsson et al., 2001]. For Western blotting, cultures were treated as above followed by immunoblotting with 4F12 monoclonal anti-ASGP-2 antibody. Western blot analysis provided similar observations—no inhibition of Muc4/SMC precursor processing in the presence of 25 mM NH_4Cl (Fig. 4B). Mobility of the ASGP-2 subunit was also slightly affected in treated cultures. These results suggest that the mechanisms of Muc4/SMC and MUC2 cleavage are not the same.

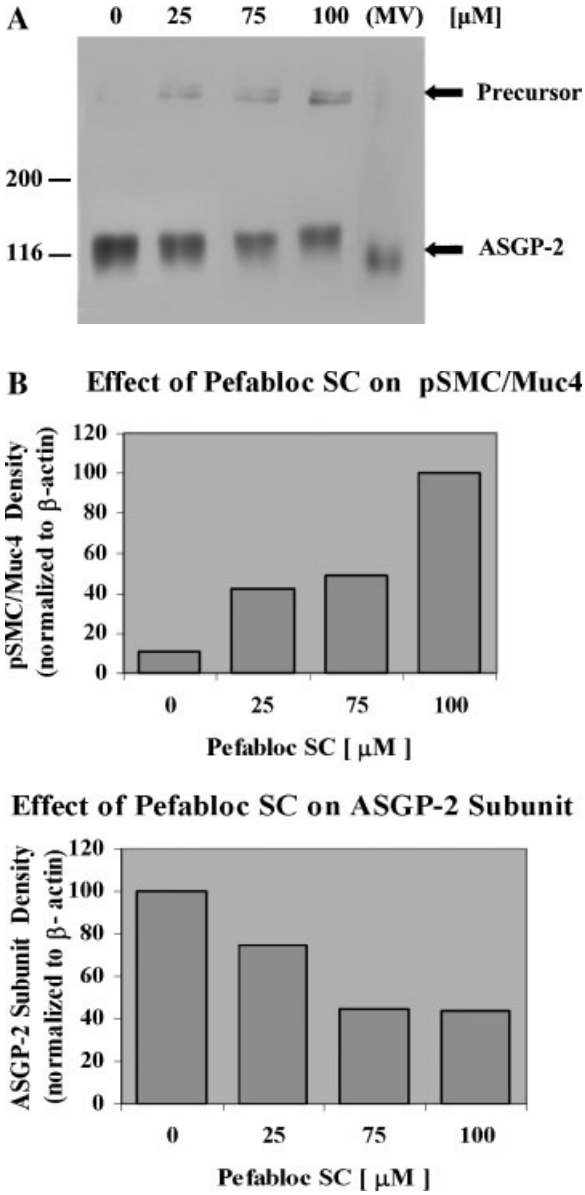

Fig. 3. Effect of Pefabloc SC on Muc4/SMC precursor and processed ASGP-2. **A**: Immunoblot of transfectant A375-Rep5 cells treated with or without Pefabloc SC (0–100 μM) for a period of 24 h. Lysate was analyzed by anti-ASGP-2 mAb (4F12). **B**: Graphic representation of A. pMuc4/SMC and ASGP-2 protein expression were normalized to β-Actin and to 100% for the 100 and 0 μM inhibitor concentration of the precursor and ASGP-2 subunit, respectively. The slight shifts in mobility in the 75 and 100 μM samples are due to a gel distortion. +sample is from ascites cell microvilli, positive control.

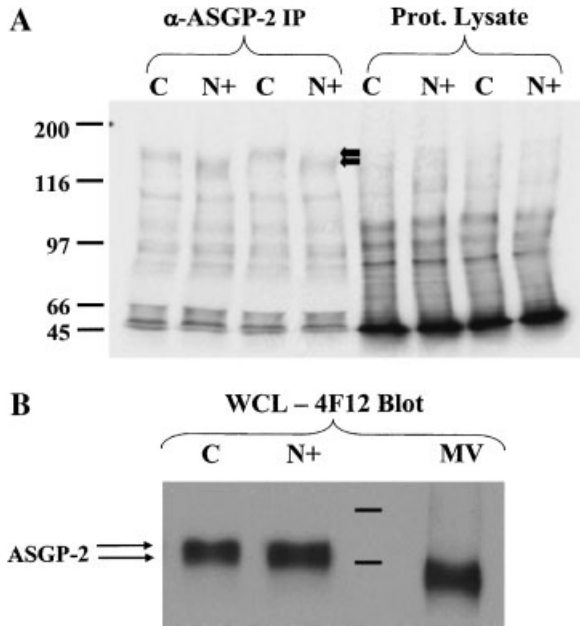

Fig. 4. Neutralization of the secretory pathway does not inhibit Muc4/SMC processing. **A**: Transfectant A375-Rep5 cells were pre-incubated with or without 25 mM NH_4Cl for 12 h followed by 8-h labeling period (with NH_4Cl). Lysates were immunoprecipitated with anti-ASGP-2 pAb for analysis by SDS–PAGE and fluorography. Control, no inhibitor; Arrows, position of ASGP-2 bands. **B**: Same experimental conditions as in A. Western blot with anti-ASGP-2 mAb, 4F12. C, Control; N+, NH4Cl; MV, microvilli, positive control.

DISCUSSION

In our previous work we characterized the signaling pathways involved in regulating Muc4/SMC expression post-translationally by TGF-β and IFN-γ in mammary epithelial cells [Soto et al., 2003]. The mucin Muc4/SMC is processed into two subunits [Sheng et al., 1990], a mucin (ASGP-1) and a transmembrane subunit (ASGP-2) by this post-translational mechanism. The TGF-β pathway has been proposed to act via an unidentified protein which functions to inhibit Muc4/SMC precursor processing [Price-Schiavi et al., 2000, 2002; Soto et al., 2003]. Cleavage of the precursor form of Muc4/SMC is known to occur near the transfer from the endoplasmic reticulum to the Golgi [Sherblom and Carraway, 1980; Sherblom et al., 1980]. Since TGF-β has been postulated to act by an inhibition of a proteolytic cleavage, we conducted protease inhibitory assays with broad-based inhibitors for aspartic, caspase, cysteine, metallo, and serine proteases in an attempt to elucidate the type of protease involved. No protease has been identified for the cleavage step of the precursor form of Muc4/SMC. Of the inhibitors used, only Pefabloc SC, a mechanism-based serine protease inhibitor of the class of sulfonyl fluorides significantly inhibited Muc4/SMC processing, thereby suggesting that the cleavage enzyme is a serine

protease. Other serine protease inhibitors, PMSF and Leupeptin, did not significantly affect Muc4/SMC processing, suggesting that the specificity of the inhibitors is different. Treatment of cell cultures with a range of Pefabloc SC concentrations indicated a dose dependence. Consistent with an inhibition of precursor cleavage, we observed an increase of the precursor of Muc4/SMC with increasing concentrations of Pefabloc SC concomitant with a decrease in levels of processed ASGP-2 subunit.

Investigation of MUC2, a gel-forming mucin with a similar cleavage site to that of Muc4/SMC [Lidell et al., 2003] has suggested an autocatalytic cleavage mechanism that is triggered in the late secretory pathway at low pH (<6.0). When the secretory pathway is neutralized with 25 mM NH_4Cl, MUC2 cleavage is inhibited. However, this explanation for the cellular cleavage of Muc4/SMC is problematic for two reasons. First, we have previously shown that the cleavage of Muc4/SMC precursor occurs early in the transit to the cell surface [Sheng et al., 1990] in compartments that are less acidic. Second, we have shown that this processing step can be inhibited by TGF-β [Price-Schiavi et al., 2000; Soto et al., 2003] via the Smad pathway in a process that exhibits no apparent alterations of the glycosylation of ASGP-2. It is unlikely that the TGF-β is affecting the acidity of the organelles processing Muc4/SMC and more likely that it is affecting the expression of a protease or protease inhibitor involved in Muc4/SMC precursor cleavage. In our present study with Muc4/SMC, no inhibition of Muc4/SMC processing was observed under similar conditions by treatment with NH_4Cl. The differences found between MUC2 and Muc4/SMC processing probably result from differences in the sequences flanking the cleavage site, making the MUC2 resistant to cleavage in the early compartment, then susceptible to acid cleavage later in the pathway. We propose that Muc4/SMC is cleaved by a yet unidentified serine protease. Since Muc4/SMC is cleaved before reaching acidic compartments, its cleavage is not subjected to the same conditions as MUC2.

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