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## TGF $\beta$ Regulation of Membrane Mucin Muc4 via Proteasome Degradation

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### Abstract

Muc4 is a heterodimeric membrane mucin implicated in epithelial differentiation and tumor progression. It is expressed from a single gene as a 300 kDa precursor protein which is cleaved in the endoplasmic reticulum to its two subunits. Our previous work has shown that Muc4 is regulated by TGF $\beta$ , which represses the precursor cleavage. Working with Muc4-transfected A375 tumor cells, we now show that Muc4 undergoes proteasomal degradation. Proteasome inhibitors prolong the life of the precursor, shunt the Muc4 into cytoplasmic aggresomes, increase the level of Muc4 associated with the endoplasmic reticulum chaperones calnexin and calreticulin and increase the levels of ubiquitinated Muc4. Most importantly, proteasome inhibitors repress the TGF $\beta$  inhibition of Muc4 expression. These results suggest a model in which TGF $\beta$  inhibits precursor cleavage, shunting the precursor into the proteasomal degradation pathway. Thus, the cells have evolved a mechanism to use the quality control pathway for glycoproteins to control the quantity of the protein produced.

### INTRODUCTION

The membrane mucin Muc4 has been implicated in tumor progression and resistance to therapies by several mechanisms. First, the mucin can act as an anti-adhesive, sterically blocking the approach of other cells [Komatsu et al., 1997,1999] and even repressing antibody binding to the cell surface of tumors [Komatsu et al., 1999;Price-Schiavi et al., 2002]. By this anti-recognition mechanism Muc4 on tumor cells can block immune cell killing of the tumor cells [Komatsu et al., 1999] and inhibit Herceptin binding and action [Price-Schiavi et al., 2002;Nagy et al., 2005]. Second, Muc4 can bind as an unorthodox intramembrane ligand via an EGF-like domain to the receptor tyrosine kinase ErbB2 [Carraway et al., 1999]. Complex formation between Muc4 and ErbB2 occurs soon after the two proteins are synthesized and can influence the localization, phosphorylation and signaling of the ErbB2 in both polarized epithelial [Ramsauer et al., 2006] and tumor [Funes et al., 2006] cells. In conjunction with the ErbB3 ligand neuregulin, Muc4 can promote signaling through the phosphoinositide 3-kinase/Akt pathway which supports tumor progression [Funes et al., 2006]. Finally, Muc4 can act as an anti-apoptotic [Komatsu et al., 2001;Hu et al., 2003], promoting the survival of tumor cells and repressing their susceptibility to therapeutic drugs [Hu et al., 2003].

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The multiple roles of Muc4, protecting normal epithelia from external insults, but also promoting tumor progression, suggest that the mucin must be tightly regulated in epithelia, but that the regulation is lost in tumors. In the mammary gland we have shown that Muc4 is regulated post-translationally by TGF $\beta$  [Price-Schiavi et al., 1998,2000;Soto et al., 2003]. This regulation is lost in mammary tumors as they lose their responsiveness to the growth factor. Our previous work has shown that TGF $\beta$  regulates Muc4 via the SMAD pathway [Soto et al., 2003]. What is unclear is how this regulation acts on the mucin. In previous studies on mammary epithelial cells we showed that TGF $\beta$  inhibits Muc4 processing [Price-Schiavi et al., 2000;Soto et al., 2003]. Rat Muc4 is composed of two subunits, the mucin subunit ASGP-1 and the transmembrane subunit ASGP-2, encoded by a single gene [Sheng et al., 1992]. The 9 kb transcript from this gene [Sheng et al., 1992;Wu et al., 1994] is translated as a 300 kDa N-glycosylated precursor [Sheng et al., 1990]. This precursor pMuc4 is cleaved into the two subunits early in its transit to the cell surface, before O-glycosylation of ASGP-1 [Sheng et al., 1990]. TGF $\beta$  acts by repressing the cleavage of the precursor to the mature heterodimeric form [Price-Schiavi et al., 2000].

This scenario raised an interesting possibility, that failure to cleave the precursor results in its transit to the proteasome and degradation. To test this mechanism, we used a tumor cell line developed previously, which has been stably transfected with a tetracycline-regulated Muc4 construct. Using that cell line, we demonstrated that TGF $\beta$  blocks the expression of both Muc4 and its precursor. We further demonstrated proteosomal degradation of Muc4 using proteasome inhibitors and that these inhibitors can block TGF $\beta$  downregulation of Muc4. These findings support a novel regulatory mechanism in which TGF $\beta$  represses cleavage of the Muc4 precursor, which is then targeted for degradation by the proteasome.

## MATERIALS AND METHODS

### Cell lines and cell cultures

A375 melanoma cells stably transfected with a tetracycline-responsive inducible construct for Muc4 were previously described [Komatsu et al., 1997]. Cells were cultivated to 70% confluence in DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 0.3 mg/ml hygromycin, 0.8 mg/ml G418 and 2  $\mu$ g/ml of tetracycline. To induce expression of Muc4, the cells were washed three times with antibiotic-free medium and then cultivated with tetracycline-free medium for two subsequent days before treatment. Fisher 344 rats were used in accordance with the National Institutes of Health Guide and Association for Research in Vision and Ophthalmology for the Care and Use of Laboratory Animals. The MAT-B1 subline ascites cells of the 13762 rat mammary adenocarcinoma were maintained and isolated from peritoneal fluid of 4 to 6 month old female rats as previously described [Carraway et al., 1976]. Shortly after isolation from the peritoneal cavity, cells were washed three times with warmed (37°C) phosphate-buffered saline (PBS) and collected by centrifugation at 800  $\times$  g. Cells were resuspended in medium and plated on plastic six well cell culture dishes. Rat corneal epithelial cells were isolated from Fisher 344 rats and maintained in culture as previously described [Lomako et al., 2005].

### Antibodies

Antibodies used during this study were developed in our laboratory: mAb 4F12 recognizing an extracellular epitope of ASGP-2 [Rossi et al., 1996], polyclonal C-pep raised in rabbits and directed against a peptide from the C-terminal cytoplasmic domain of ASGP-2 [Rossi et al., 1996] and polyclonal anti-ASGP-2 against isolated ASGP-2 [Sheng et al., 1989]. Polyclonal antiubiquitin and monoclonal anti- $\beta$ -actin antibodies were from Sigma. Anti-calnexin and anti-calreticulin were from Stresgen Bioreagents.

## Immunoblotting

To analyze cellular proteins by immunoblotting, cell collection, lysis and immunoblotting were performed as described previously [Komatsu et al., 1997]. Briefly, collected cells were solubilized for 5 min in boiling 2% sodium dodecyl sulfate (SDS). Extracts were clarified by centrifugation at  $14,000 \times g$  for 10 min, and 30  $\mu\text{g}$  protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were probed for 2 h with primary antibodies diluted in 1% bovine serum albumin (BSA)-Tris-buffered saline/0.02% Tween-20 (TTBS), washed three times with TTBS and subsequently incubated for 1 hr with appropriate secondary antibody-horseradish peroxidase-conjugates (Pierce, Rockford, IL) followed by protein visualization by the Renaissance™ Enhanced Chemiluminescence Kit (NEN Life Science Products, Inc., Boston, MA). In each case  $\beta$ -actin was used as a control for protein loading.

## Immunoprecipitation

For immunoprecipitation the ubiquitinated or radioactively labeled protein ice-cold-PBS-washed cells were extracted for 5 min with boiling modified RIPA buffer (1% SDS, 150 mM NaCl, 1% Nonidet p-40, 0.5% deoxycholate, 50 mM Tris/HCl, pH 8.0) [Rossi et al., 1996]. Extracts were clarified by centrifugation and diluted 10 fold with SDS-free RIPA buffer containing a cocktail of proteinase inhibitors (Sigma-Aldrich). For complex formation between Muc4 and ER chaperones, cells were lysed in RIPA buffer (0.1% SDS, 150 mM NaCl, 1% Nonidet p-40, 0.5% deoxycholate, 50 mM Tris/HCL, pH 8.0, containing a cocktail of proteinase inhibitors). 1 ml of extract was added to 30  $\mu\text{l}$  protein G/A-agarose beads with bound antibodies. Samples were rotated overnight at  $4^\circ\text{C}$  and washed five times with cold RIPA buffer, and immunoadsorbed proteins were eluted for 5 min with boiling SDS-PAGE sample buffer. Immunoprecipitated proteins were analyzed by immunoblotting with appropriate antibodies.

## Immunofluorescence analysis

Adherent Muc4-transfected A375 cells were grown on 6 mm Transwel-Clear™ inserts (Corning, Costar) for 1 day and then for 48 hours after induction of Muc4 expression. Cells were washed three times with Dulbecco's PBS, pH 7.4, fixed in 4% formaldehyde in PBS for 20 min and, when indicated, permeabilized by two five minute treatments with 0.2% Triton X-100 in PBS. Aldehyde groups were quenched with 50 mM ammonium chloride and, after one 5 min wash with PBS and two subsequent washes with 1% BSA in PBS, cells were incubated for 1 h with indicated primary antibodies diluted in BSA-PBS. The cells were rinsed for 5 min twice with BSA-PBS, twice with PBS and incubated in the dark for 1 h with specific secondary antibodies coupled to Alexa Fluor 488 or Texas Red (Molecular Probes, Eugene, OR) and subsequently washed three times with PBS. Membranes containing the cells were excised from the inserts and mounted with a Prolong Antifade Kit (Molecular Probes) on microscope glass slides. To distinguish between membrane and intracellular localization of Muc4, nonpermeabilized cells were labeled first with mAb 4F12. The cells were then permeabilized, and intracellular Muc4 protein was labeled. The fluorescence labeling was examined by confocal microscopy using an LSM 510 microscope (Carl Zeiss, GmbH, Germany) equipped with two laser sources. Cells were analyzed using 100x oil immersion objectives. The images were collected and processed using LSM 510 software obtained from Zeiss.

## Pulse-chase labeling of A375 cells expressing Muc4

A375 melanoma cells stably transfected with tetracycline-responsive inducible Muc4 vector were grown to 70% confluence. Two days after induction of the expression of Muc4, cells were washed three times with PBS, starved for 2 h in Cys/Met-free DMEM essential

medium supplemented with 100 IU/ml penicillin, 0.3 mg/ml hygromycin, and 0.8 mg/ml G418 (starvation medium) and subsequently incubated in starvation medium containing 100  $\mu$ Ci/ml of  $^{35}$ S EXPRESS Protein Labeling Mix (NEN Life Science). Labeled cells were washed three times with ice-cold PBS and lysed in modified RIPA buffer. Extracts were clarified by centrifugation at 10,000 rpm, and cell lysates were immunoprecipitated with polyclonal Ab against ASGP-2. Protein released from the polyclonal Ab-A/G agarose beads by boiling for 5 min with SDS-sample buffer was analyzed by SDS-PAGE and fluorography.

## RESULTS

### TGF $\beta$ represses expression of Muc4 in A375 tumor cells

Our previous studies on the repression of Muc4 expression by TGF $\beta$  were done on primary rat mammary epithelial cells [Price-Schiavi et al., 1998,2000;Soto et al., 2003]. However, studies on these primary cells are very time-consuming and expensive. To further investigate the mechanism by which TGF $\beta$  acts on Muc4, we have turned to a cell line, A375 melanoma, transfected with Muc4, in which the Muc4 is under the regulation of a tetracycline-dependent promoter [Komatsu et al., 1997]. To determine whether TGF $\beta$  represses Muc4 expression in the transfected A375 cells in a similar manner to that previously observed with the mammary epithelial cells, we treated the A375 cells expressing Muc4 with TGF $\beta$  for 20 hr at concentrations similar to those used with the mammary cells. As shown in Figure 1A, TGF $\beta$  has a pronounced inhibitory effect on Muc4 expression in the A375 cells. Moreover, the delayed timing of this effect (Figure 1B) is consistent with our previous observations that TGF $\beta$  is acting on the precursor of Muc4 [Price-Schiavi et al., 2000] and its effect is only observed when the Muc4 has had sufficient time to undergo degradation. This mechanism of action of TGF $\beta$  on Muc4 synthesis is further supported by pulse-chase analyses, which show that label incorporation into the transmembrane subunit of Muc4 is blocked and that precursor is degraded in the TGF $\beta$ -treated cells (Figure 1C).

### Muc4 degradation by proteosomal pathway

Since precursor cleavage of Muc4 is suggested to occur in the endoplasmic reticulum [Sheng et al., 1990], we propose that TGF $\beta$  regulation of Muc4 might involve proteosomal degradation. To determine whether Muc4 undergoes significant proteosomal degradation, we have treated two different cell types expressing Muc4 with proteosomal inhibitors and analyzed their Muc4 content. As noted above, one of these, the A375, cells have been transfected with Muc4 to induce overexpression. The MAT-B1 13762 rat ascites mammary adenocarcinoma cells have endogenous levels of Muc4. As shown in Figure 2A, treatment of the cells with two different proteasome inhibitors resulted in increased Muc4 expression, indicating that Muc4 is subject to proteasome degradation. These results show that proteasome degradation of Muc4 is occurring in cancer cells with both endogenous and transfected Muc4. A similar effect has also been shown in isolated corneal epithelial cells (data not shown). Importantly, pulse-chase analyses of the A375 cells indicated that proteasome inhibitor repressed the degradation of the Muc4 precursor, as shown previously in mammary epithelial cells [Price-Schiavi et al., 2000] (Figure 2B).

Previous studies by others have shown that inhibition of proteasome degradation results in proteins from the endoplasmic reticulum being accumulated in aggresomes in the cell cytoplasm [Johnston et al., 1998; Kopito 2000]. Therefore, we measured incorporation of Muc4 into aggresomes analyzed by two color confocal microscopy. Cell surface Muc4 was first labeled on intact cells by a green-labeled antibody against the extracellular domain of Muc4. The cells were then permeabilized, and the cytoplasmic Muc4 was labeled with a red-labeled antibody against the cytoplasmic domain of Muc4. Figure 3 shows that cells treated

with proteasome inhibitor have a substantial accumulation of Muc4 in cytoplasmic structures.

### Endoplasmic reticulum degradation pathway for Muc4

Glycoproteins undergoing degradation in the endoplasmic reticulum interact with chaperones which are involved in the processing of N-linked oligosaccharides [Ellgaard and Helenius, 2003]. To investigate the components which interact with Muc4, we treated the A375 cells with the mannosidase inhibitor deoxymannojirimycin (DMJ) to promote Muc4 degradation by the proteosomal degradation pathway, as shown in Figure 4A. The effect of the DMJ on the Muc4 degradation was reversed by proteasome inhibitor. To determine whether the chaperones calnexin and calreticulin are involved in the Muc4 degradation, cells were treated with or without the mannosidase inhibitor deoxymannojirimycin (DNJ), then lysed and immunoprecipitated with anti-calnexin or anti-calreticulin. Immunoblots of the immunoprecipitates with anti-Muc4 showed clearly that interaction of Muc4 with both of these chaperones is increased by the DNJ treatment (Figure 4B).

### Ubiquitination of Muc4

Proteosomal degradation of glycoproteins from the endoplasmic reticulum requires that they be ubiquitinated for recognition by the proteasome [Weissman, 2001; Pickart and Cohen, 2004]. Muc4 ubiquitination was analyzed by sequential immunoprecipitation and immunoblotting with anti-ubiquitin and anti-Muc4 in lysates from A375, MAT-B1. Levels of ubiquitinated Muc4 are increased in both tumor cell types by treatment with proteasome inhibitors (Fig. 5), though the effects appear less pronounced in the ascites cells. A similar effect was observed in isolated corneal epithelial cells (data not shown)

### Proteasome inhibitor reverses the effect of TGF $\beta$ on Muc4 expression

If TGF $\beta$  is repressing Muc4 expression by enhancing proteosomal degradation, then proteasome inhibitor should block the TGF $\beta$  effect and restore the Muc4 expression. Figure 6A shows that the proteasome inhibitor PSI is effective in preventing the loss of Muc4 due to TGF $\beta$ .

## DISCUSSION

Proteosomal degradation is used for both quality and quantity control for cellular proteins [Ellgaard and Helenius, 2003; Wojcikiewicz, 2004]. The need for quality control for Muc4 is obvious. It is a complex glycoprotein with numerous disulfides and multiple domains, such as the epidermal growth factor domains [Sheng et al., 1992], which require precise folding. Quantity control is also important for membrane mucins. The anti-adhesive properties of these molecules are critical for protection of epithelial cell surfaces, but overproduction can potentially compromise cell-cell interactions [Komatsu et al., 1997, 1999]. Membrane mucin overexpression is frequent in some carcinomas, contributing to their dissemination and metastasis [Komatsu et al., 2000]. We have shown previously that TGF $\beta$  is a key regulator of Muc4 in mammary epithelial cells [Price-Schiavi et al., 1998, 2000; Soto et al., 2003]. This regulatory mechanism is lost in mammary tumors with tumor progression due to loss of responsiveness to TGF $\beta$  [Price-Schiavi et al., 2000]. Our current work on the A375 and ascites tumor cells suggests that this is a general mechanism for regulating Muc4. An important question is how TGF $\beta$  acts on Muc4. Our previous work suggested that TGF $\beta$  repressed processing of the precursor of Muc4 to its two subunits in the endoplasmic reticulum. Our present work indicates that the precursor is then targeted to the proteasome for proteosomal degradation (Figure 6B), possibly because the precursor is unable to fold correctly without cleavage. These results suggest that a quantity control mechanism for Muc4 has evolved from the general quality control mechanism.

In summary, our work provides the first evidence for a role for proteosomal degradation in regulating mucin production in cells, offering a route for both the quality and quantity control necessary to maintenance of the protective epithelial barrier.

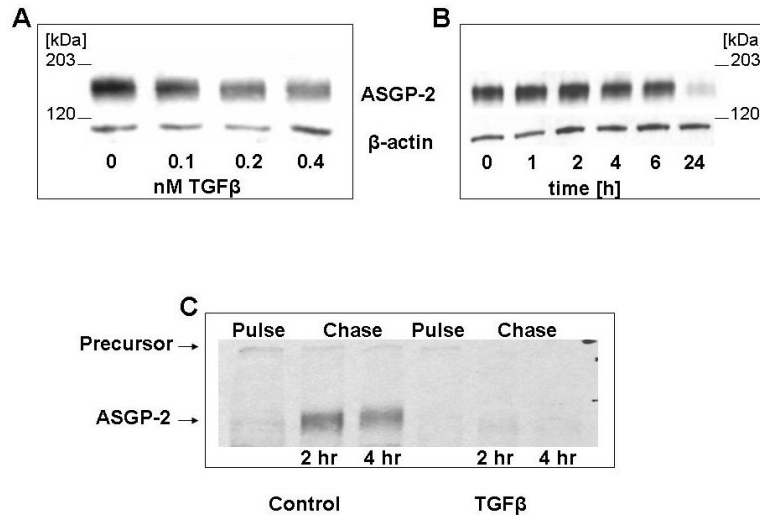
## Acknowledgments

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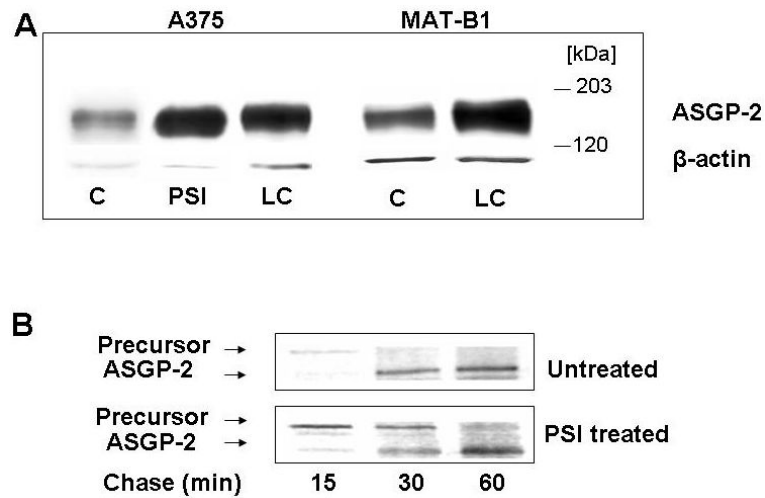
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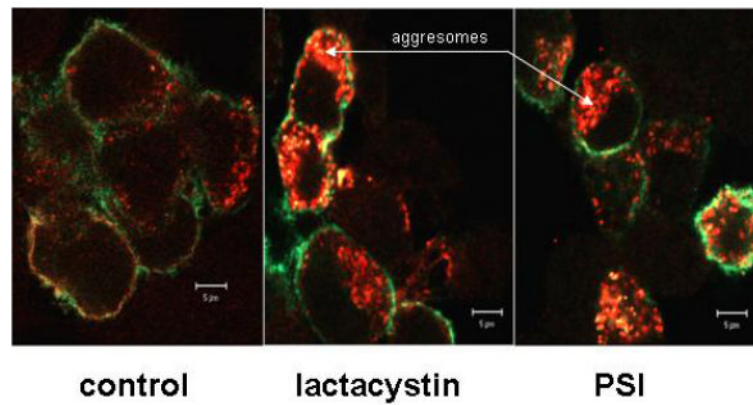


**Fig. 1.** TGFβ repression of Muc4 expression in tumor cells. (A) A375 cells were treated with tetracycline for 48 hr to activate Muc4 expression, then treated with TGFβ at the concentrations shown for 24 hr. Muc4 was analyzed by immunoblotting. (B) Muc4-expressing A375 cells were treated with 0.4 nM TGFβ for the periods shown, then analyzed by immunoblotting. (C) A375 cells with or without TGFβ treatment were pulse-chase labeled for the periods shown and lysed for immunoprecipitation. The lysates were analyzed by SDS PAGE and fluorography.



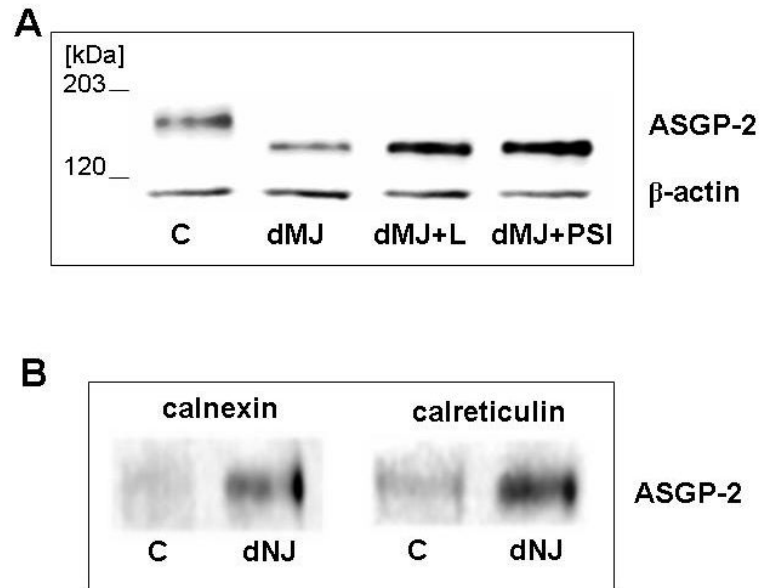
**Fig. 2.**

Effect of proteasome inhibitors on Muc4 in tumor cells. (A) A375 and 13762 MAT-B1 cell cultures were treated for 4 hours in medium containing 0.01  $\mu$ M PSI or 5  $\mu$ M lactacystin (LC) and analyzed by immunoblotting. (B) A375 cells with or without PSI treatment were pulse-chase labeled for the periods shown and lysed for immunoprecipitation. The lysates were analyzed by SDS PAGE and fluorography.

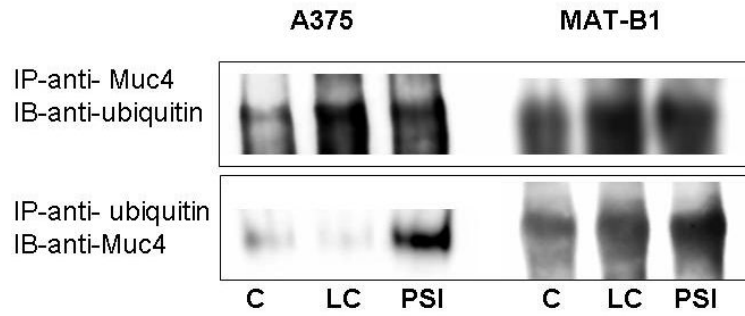


**Fig. 3.**

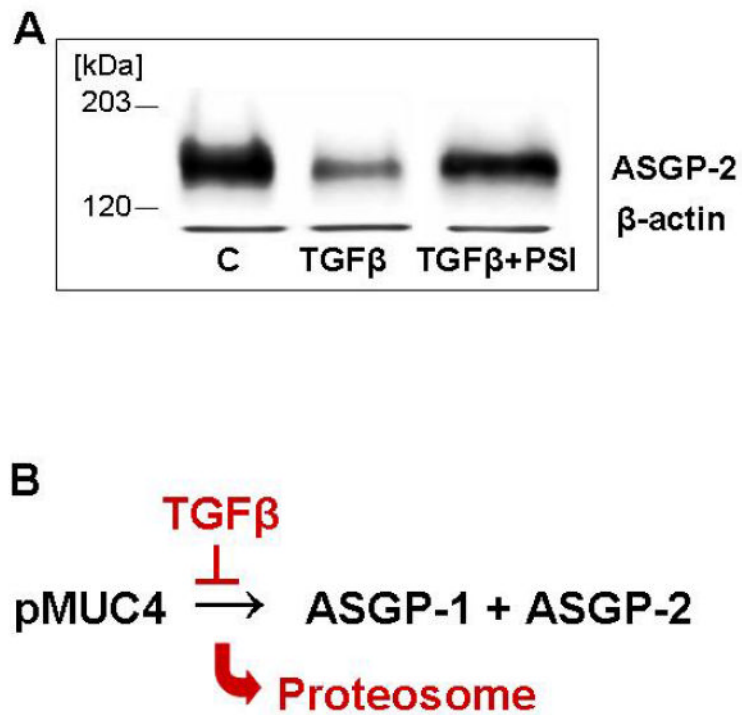
Proteasome inhibition relocalizes Muc4 in A375 cells to intracellular compartments. Two-color immunofluorescence analysis. A375 cells were grown in tetracycline-containing medium for one day after seeding on Transwell-Clear inserts and then on tetracycline-free medium for the next two days (cells “on”). Cells were then treated with 5 µM lactacystin or 0.01 µM PSI for 4 h. Cell surface Muc4 was labeled on non-permeabilized cells with 4F12 mAb directed to an extracellular epitope of Muc4, followed by anti-mouse Alexa-Fluor (green). Subsequently, cells were permeabilized with 0.2% Triton X100, and intracellular Muc4 was labeled with polyclonal anti C-pep antibody followed by Alexa-Texas Red anti-rabbit antibody (red).



**Fig. 4.** Effect of mannosidase inhibitors on Muc4 proteosomal degradation and association with ER chaperones. (A) A375 cells were grown overnight in the presence of 100  $\mu$ g/ml deoxymannojirimycin (dMJ) and treated for an additional 4 h in the presence of proteasome inhibitors. SDS-extracted proteins were subjected to SDS-PAGE and immunoblotting with 4F12 mAb. (B) A375 cells were grown in the presence of dNJ (0.5 mM) and analyzed for chaperone-Muc4 complexes by immunoprecipitation with antibodies against the chaperones. The immunoprecipitates were analyzed by immunoblotting with anti-Muc4.



**Fig. 5.** Muc4/SMC is ubiquitinated in A375 cells. Protein from RIPA extracts of A375 or 13762 MAT-B1 ascites cells treated with or without proteasome inhibitors was subjected to sequential immunoprecipitation and immunoblot analyses with anti-ubiquitin and anti-Muc4. (A) Lysates immunoprecipitated with anti-ASGP-2 polyclonal antibody coupled to A/G-agarose and immunoprecipitates immunoblotted with anti-ubiquitin antibody. (B) Lysates immunoprecipitated with anti-ubiquitin antibody coupled to A/G-agarose and immunoprecipitates immunoblotted with 4F12 mAb.



**Fig. 6.** Reversal of TGF $\beta$  inhibition of Muc4 expression by proteasome inhibitor. (A) A375 cells treated with or without TGF- $\beta$  and proteasome inhibitor were analyzed for Muc4 by immunoblotting. (B) Model for TGF $\beta$  regulation of Muc4 by proteosomal degradation.