SMAD2 and SMAD7 Involvement in the Post-translational Regulation of Muc4 via the Transforming Growth Factor-β and Interferon-γ Pathways in Rat Mammary Epithelial Cells*

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Muc4/sialomucin complex (SMC) is a heterodimeric glycoprotein complex derived from a single gene that is post-translationally processed into mucin (ASGP-1) and transmembrane (ASGP-2) subunits. Muc4/SMC is tightly regulated in the rat mammary gland, low in the virgin, increased during pregnancy and lactation, and overexpressed in some aggressive mammary tumors. Investigations of primary rat mammary epithelial cells (MEC) have shown that Muc4/SMC expression is post-translationally regulated through inhibition of Muc4/SMC precursor processing by transforming growth factor-β (TGF-β). Localization studies suggest that TGF-β inhibition of Muc4/SMC expression is mediated through SMAD2, a TGF-β effector that, when activated, functions as a transcription factor. SMAD2 antisense oligonucleotide blocks the inhibition of Muc4/SMC expression by TGF-β. The TGF-β effect on Muc4/SMC expression is repressed by interferon-γ (IFN-γ). IFN-γ treatment of MEC activates and relocates signal transducer and activator of transcription-1 (STAT-1) to induce an inhibitor SMAD, SMAD7. SMAD7 antisense oligonucleotide prevents IFN-γ from blocking the TGF-β inhibition of Muc4/SMC expression. These results suggest that TGF-β regulates Muc4/SMC expression via the SMAD pathway by a transcriptional effect on a protein in the Muc4/SMC processing step, possibly the protease that cleaves the precursor.

Muc4/SMC1 is a heterodimeric glycoprotein complex derived from a single gene that is post-translationally processed into two subunits (1), a mucin and a transmembrane subunit (2, 3). The mucin subunit ASGP-1 is a high molecular weight sialomucin that is highly O-glycosylated. The transmembrane subunit ASGP-2 is N-glycosylated and has a molecular mass of ~120 kDa. Transfection studies have shown that Muc4/SMC is a multifunctional protein with anti-adhesive (4), anti-recognition (5), and signal regulation functions (6, 7), all of which are important characteristics of both normal epithelia and tumors. Immunocytochemical analyses show the presence of Muc4/SMC in a number of different vulnerable epithelia, including those of the female reproductive tract, airway, ocular surface, and mammary gland (8). Muc4/SMC has also been studied in both rat and human mammary carcinomas, where its expression appears to be related to tumor aggressiveness (9). Thus, it is of considerable interest to understand the regulation of Muc4/SMC expression in the mammary gland and its tumors.

In previous studies we showed that Muc4/SMC is up-regulated during pregnancy in the rat mammary gland (10). A major factor involved in this regulation appears to be TGF-β (10, 11), which inhibits Muc4/SMC expression by blocking processing of the Muc4/SMC precursor to its two subunits (11). Interestingly, this inhibitory effect of TGF-β on Muc4/SMC expression is lost in rat mammary adenocarcinoma cells (10), consistent with the loss of TGF-β responsiveness in many human breast cancers (12). TGF-β is a potent inhibitor of a variety of cell types including epithelial, endothelial and hematopoietic cells (13). Many tumor cells, however, become refractory to the inhibitory effects of TGF-β, primarily due to defects in the TGF-β signaling pathway (13, 14). TGF-β signals through a receptor serine-threonine kinase that phosphorylates and activates the transcription factors SMAD2 and SMAD3 (15). SMADs can be divided into three classes. The receptor-regulated, or R-SMADs (SMAD2 and SMAD3) can form a heterodimeric complex with a second class, or co-SMAD, of which SMAD4 is the only member. This complex is translocated to the nucleus where it can directly bind to DNA or associate with other transcription factors to regulate transcription. The third class, the inhibitory SMADs, or I-SMAD (SMAD6 and SMAD7), counteract the effect of the R-SMADs, antagonizing TGF-β signaling (13–15).

One of the mechanisms for regulating I-SMAD effects is via the cytokine IFN-γ (16). IFN-γ is the only member of the Type II class of interferons (17) and exerts its biological effects by binding to its cognate receptor, the Type II IFN receptor. The Type II IFN receptor is associated with two tyrosine kinases, Janus kinase-1 and -2. Upon binding of IFN-γ to the receptor, Jak-1 and/or Jak-2 are activated and phosphorylate the Type II IFN receptor. The phosphorylated receptor provides a docking site for a STAT, which then acts as a substrate for Jak (1 or 2) to undergo phosphorylation (17). The phosphorylated STAT forms a homodimer that can translocate to the nucleus to regulate gene transcription (18). TGF-β and IFN-γ have been shown to have antagonistic effects on cellular functions (16,

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‡The abbreviations used are: SMC, sialomucin complex; TGF, transforming growth factor; ASGP, asialoglycoprotein; MEC, primary mammary epithelial cells; FBS, fetal bovine serum; BSA, bovine serum albumin; STAT, signal transducer and activator of transcription; IFN, interferon; Jak, Janus kinase; ITS, insulin, transferrin, and sodium selenite; mAb, monoclonal antibody; pAb, polyclonal antibody; DAPI, 4′,6-diamidino-2-phenylindole; S-oligo(s), phosphorothioate single-stranded DNA oligonucleotide(s).
Recent studies have shown that IFN-γ acts on the TGF-β pathway via Jak-1 and STAT-1 to induce SMAD7 expression, which inhibits TGF-β-R-SMAD phosphorylation and its downstream events (16). In the present study we have characterized the post-translational mechanism involved in the regulation of Muc4/SMC by TGF-β. TGF-β is shown to activate SMAD2, but not SMAD3, in the TGF-β pathway. TGF-β inhibition of Muc4/SMC expression is repressed by IFN-γ. The IFN-γ effect occurs through the up-regulation of SMAD7 via the STAT-1 pathway. These results suggest that TGF-β blocks Muc4/SMC expression through an indirect transcriptional effect, possibly on the enzyme involved in the cleavage of the Muc4/SMC precursor.

EXPERIMENTAL PROCEDURES

Materials—Anti-ASGP-2 polyclonal antiserum was prepared against purified ASGP-2. The mouse monoclonal antibody 4F12, which was elicited using purified Muc4/SMC, recognizes an epitope in the N-terminal 53 amino acids of ASGP-2. TGF-β and IFN-γ were purchased from R & D Systems, Inc. (Minneapolis MN). Cell culture materials were purchased from Life Technologies, Inc. (Paisley, UK). FBS was purchased from Roche Diagnostics. SMAD7 antibody was ordered from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). SMAD7 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-β-actin antibody was purchased from Sigma. Insulin-transferrin-selenium (ITS) supplement was purchased from Roche Diagnostics.

Primary Cell Culture and Cell Lines—Primary mammary epithelial cells (MEC) were obtained from the mammary glands of 344 Fischer rats (NCR) as described previously (10). Cell culture materials were purchased from Life Technologies, Inc. (Paisley, UK). TGF-β and IFN-γ were obtained from Invitrogen. Phosphorothioate single-stranded DNA oligonucleotides were obtained from Sigma-Genosys (Woodlands, TX). DAPI was purchased from Pierce. SMAD2 and SMAD3 and STAT antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA). SMAD7 antibody was ordered from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse monoclonal antibody 4F12, which was elicited using purified Muc4/SMC, recognizes an epitope in the N-terminal 53 amino acids of ASGP-2. TGF-β and IFN-γ were purchased from R & D Systems, Inc. (Minneapolis MN). Cell culture materials were purchased from Life Technologies, Inc. (Paisley, UK). FBS was purchased from Roche Diagnostics. SMAD7 antibody was ordered from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-β-actin antibody was purchased from Sigma. Insulin-transferrin-selenium (ITS) supplement was purchased from Roche Diagnostics.

Immunofluorescence—MEC were cultured on four-well chamber slides. Cells were washed in phosphate-buffered saline followed by incubation in 4% paraformaldehyde. Cells were permeabilized in 0.1% Triton X-100. Primary and secondary antibodies were diluted in 3% FBS/phosphate-buffered saline. Between washes, cells were exposed to each antibody for a period of 1 h. DAPI (1 µg/ml) stain was used to determine nuclear localization. Coverslips were mounted using Permount antifade (Molecular Probes).

Immunoblotting—Cell lysates were electrophoresed under reducing conditions using 8% polyacrylamide gels and transferred onto nitrocellulose membranes. Nitrocellulose membranes were blocked with 3% bovine serum albumin/TBS-buffered saline with 0.5% Tween 20 (TTBS) for 1 h to overnight and washed with 1% bovine serum albumin/TBS wash buffer. Signals were detected with the Renaissance™ enhanced chemiluminescence kit (PerkinElmer Life Sciences).

RESULTS

Effect of TGF-β and IFN-γ on Muc4/SMC Protein Expression—Muc4/SMC has been previously shown to be regulated by TGF-β post-translationally (10, 11). The aim of the current study was to define the mechanism previously postulated for post-translational regulation of Muc4/SMC. Previous studies have shown that IFN-γ can interfere with TGF-β effects (16, 19). To determine whether IFN-γ can block the inhibitory effect of TGF-β on Muc4/SMC expression, we used primary MEC that were cultured with the presence or absence of TGF-β (200 pg) and/or IFN-γ (100 ng/ml) for 24 h. After the 24-h period, cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 monoclonal antibody, 4F12, which recognizes the membrane subunit of Muc4/SMC protein, and anti-β-actin monoclonal antibody as loading control. Immunoblot analysis indicated that Muc4/SMC expression was decreased by 50% in MEC treated with TGF-β when compared with MEC cultured without TGF-β (Fig. 1). IFN-γ alone had little or no effect on Muc4/SMC expression levels. However, MEC treated both with IFN-γ and TGF-β showed similar levels of Muc4/SMC expression to that of the non-treated MEC, indicating that IFN-γ can repress the TGF-β effect on Muc4/SMC expression.

Dose Response of IFN-γ Effect in Normal MEC—IFN-γ was able to block the TGF-β effect on Muc4/SMC at a concentration of 100 ng/ml (see Fig. 1). To determine whether the IFN-γ effect is dose-dependent, MEC were treated with 0, 50, 100, and 200 ng/ml IFN-γ and/or TGF-β (200 pg) for 24 h. In the 24-h period, cells were harvested, lysed, and subjected to immunoblot analysis with monoclonal antibodies directed against Muc4/SMC and β-actin. TGF-β, as expected, inhibited Muc4/SMC expression levels by about 50%, while IFN-γ alone had relatively little or no effect on Muc4/SMC levels (Fig. 2). At 50 ng/ml IFN-γ did not block inhibition of Muc4/SMC expression by TGF-β, but at 100 and 200 ng/ml IFN-γ was able to block the TGF-β effect.

These data suggest that blockade of the inhibitory effects of TGF-β on Muc4/SMC expression by IFN-γ is dose-dependent and that the balance of IFN-γ and TGF-β signals may determine the ultimate downstream effect.

Timing of TGF-β and IFN-γ Effects on Muc4/SMC Levels—To determine whether the order of addition, or timing of exposure of MEC to TGF-β or IFN-γ, were determining factors in the results obtained, we treated MEC with either TGF-β or IFN-γ for a period of 24 h. After an additional 24 h, TGF-β was added to the IFN-γ-treated MEC and IFN-γ was added to the TGF-β-treated MEC. After an additional incubation for periods up to 24 h, the cells were harvested, lysed, and subjected to immunoblot analysis with 4F12 and β-actin antibodies to determine Muc4/SMC and β-actin expression levels, respectively. When cells were treated with TGF-β first, followed by IFN-γ, Muc4/SMC levels remained similar to those in MEC treated
with TGF-β alone (Fig. 3). When cells were treated with IFN-γ first, followed by treatment with TGF-β, Muc4/SMC levels remained high, similar to those in untreated cells or those treated with IFN-γ alone. These data suggest that signaling through the TGF-β and IFN-γ pathway is persistent such that once a signaling pathway is initiated by one factor (either TGF-β or IFN-γ), signaling through the other factor cannot interfere.

Relocalization of SMAD2 by TGF-β in MEC—Signaling through TGF-β occurs via transmembrane serine-threonine kinase receptor (13–15), which activate receptor-regulated SMADs and induce their translocation to the nucleus. R-SMAD appearance in the nucleus peaks at ~45 min after TGF-β stimulation and declines after 6 h (20). To determine which R-SMAD functions in the TGF-β pathway in normal virgin mammary epithelial cells, MEC were treated with TGF-β for a period of 1 h. Cells were lysed in hypotonic lysis buffer, fractionated, and subjected to immunoblot analysis with polyclonal antibodies for SMAD2 and SMAD3 to observe translocation to the nucleus. Total SMAD2 levels were determined by the addition of the fractions (membrane, cytosolic, and nuclear) in individually treated cultures. When MEC were treated with TGF-β, SMAD2 in the nucleus increased compared with that in control cells; ~80% of total SMAD2 was found in the nuclear fraction in TGF-β-treated cells. Cells treated with both TGF-β and IFN-γ reversed the effect of the TGF-β, though not completely, yielding a similar pattern of SMAD2 localization as control non-treated MEC. Interestingly, 100% of SMAD2 protein was found in the cytoplasmic fraction when cells were treated with IFN-γ (Fig. 4A). Thus, the nuclear localization of SMAD2 is parallel to the effects on Muc4/SMC expression, though not directly correlated. SMAD3, the other receptor-regulated SMAD in the TGF-β pathway, was not translocated.

Fig. 1. Effect of TGF-β and IFN-γ on Muc4/SMC expression and processing. A, MEC were treated with TGF-β (200 pM) and/or IFN-γ (100 ng/ml) for 24 h. Cell lysates were analyzed with anti-ASGP-2 mAb (4F12) and anti-β-actin mAb (loading control). B, normalized graphical representation of the data in A. Error bars are S.D. values based on three separate experiments.

Fig. 2. Dose response of IFN-γ effect in normal MECs. MEC were treated with TGF-β (200 pM) and/or IFN-γ at 0, 50, 100, or 200 ng/ml in serum-free, ITS-supplemented medium. A, immunoblot analysis of TGF-β and IFN-γ-treated cells. Cell lysates were subjected to immunoblot analysis with anti-ASGP-2 4F12 or anti-actin mAbs. B, normalized graphical representation of the data in A. Representative example of three separate experiments is shown.

Fig. 3. Timing of the effect of IFN-γ on inhibition of Muc4/SMC levels by TGF-β. MEC were cultured with 200 pM TGF-β or 100 ng/ml IFN-γ. After another 24 h IFN-γ (100 ng/ml) was added to the TGF-β treated samples, and TGF-β was added to the IFN-γ treated samples for specified times in serum-free, ITS-supplemented medium. A, immunoblot of TGF-β- and IFN-γ-treated cells. B, normalized graphical representation of the data in A. Abbreviations: T, TGF-β; I, IFN-γ; TI, TGF-β first followed by IFN-γ; IT, IFN-γ first followed by TGF-β. Representative example of three separate experiments is shown.
in the presence of TGF-β in MEC (data not shown). To observe activation/translocation of SMAD2 in the cells, MEC were subjected to immunofluorescence analysis. MEC were treated with TGF-β and/or IFN-γ for a period of 1 h. Cells were washed, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and probed with anti-SMAD2 or anti-SMAD3 antibodies, followed by secondary antibodies conjugated to Alexa Fluor 488 and/or Alexa Fluor 594. Nuclear localization was determined by DAPI stain. These data correlated with the results obtained in the fractionation study (Fig. 4A) and supported the results obtained in the fractionation study (Fig. 4B). Under all conditions, SMAD3 localization was cytoplasmic.

**STAT-1 Is Relocalized by IFN-γ in MEC**—IFN-γ treatment of cells results in phosphorylation of STATs by Jak-1 or -2 associated with the IFN-γ receptor (16, 21, 22). To investigate which STAT(s) is activated to regulate TGF-β signaling in MEC, we analyzed STAT activation with antibodies for STATs 1a, 2, 3, 4, 5a, 5b, and 6 to observe and identify STAT proteins activated/translocated in response to IFN-γ stimulation. MEC were treated with or without IFN-γ for a period of 1 h. Cells were washed, fixed, and subjected to immunofluorescence analysis as described previously. No activation of STATs 2, 3, 4, 5a, 5b, and 6 was observed in MEC when stimulated with IFN-γ (data not shown). STAT localization was primarily cytoplasmic. In contrast, STAT-1α localization to the nucleus in MEC was observed during treatment with IFN-γ (Fig. 5). STAT-1 is considered the primary STAT activated via the Type II IFN receptor (21), which is the cognate receptor for IFN-γ. However, STAT5 and STAT3 are also known to be activated in response to IFN-γ in certain cell types (17, 21). Therefore, these data suggest that STAT-1α functions as the primary STAT effector in IFN-γ-stimulated MEC and suggest a role in the regulation of Muc4/SMC via the TGF-β-stimulated pathway.

**Requirement for SMAD2 in the Post-translational Regulation of Muc4/SMC by TGF-β**—To determine the requirement for SMAD2 in the regulation of Muc4/SMC by TGF-β, we utilized antisense technology (23–26). S-oligos corresponding to a stretch of 20 nucleotides in the 5′ region of the rat SMAD2 complementary DNA sequence were synthesized in both sense and antisense orientations. A scrambled oligonucleotide sequence functioned as an additional control. Cells were cultured to 40–50% confluence, at which time cell cultures were treated individually with 50 μM of the oligonucleotides (with Oligofectamine reagent) in serum-free and antibiotic-free medium supplemented with 5 μg/ml of ITS. After a 4–6-h incubation period serum was added to the medium and allowed to incubate for a total of 24 h. No cytokines were added to S-oligo-treated cell cultures used to determine SMAD2 protein expression. SMAD2 antisense S-oligo-treated cell cultures had a reduction of ~70%, in SMAD2 protein expression as compared with SMAD2 sense and scrambled S-oligo-treated cell cultures (Fig. 5). The results indicate that the SMAD2 S-oligos function as expected. To determine the requirement for SMAD2 in the inhibitory effect of TGF-β on Muc4/SMC expression, we treated the cell cultures with TGF-β for a period of 24 h after the initial 24-h incubation with the S-oligos. This method allows us to determine whether Muc4/SMC protein expression persists when treated with both TGF-β and the SMAD2 antisense S-oligo. If SMAD2 is required for TGF-β inhibition of Muc4/SMC, then blocking SMAD2 expression will prevent TGF-β from down-regulating Muc4/SMC expression. We observed an 80% reduction in Muc4/SMC expression in cell cultures treated with the SMAD2 sense and scrambled S-oligo, compared with SMAD2 antisense-treated cell cultures (Fig. 5B). β-Actin was used to control for protein loading. The results indicate that TGF-β was not able to inhibit Muc4/SMC expression when SMAD2 expression was blocked by the antisense method.
When SMAD2 expression was not blocked (sense and scrambled S-oligos), TGF-β suppressed Muc4/SMC expression. These data support the requirement for SMAD2 activation in regulating Muc4/SMC expression via the TGF-β-SMAD pathway (11).

Requirement for SMAD7 in the Post-translational Regulation of Muc4 by IFN-γ—SMAD6 and SMAD7 are inhibitors of TGF-β family signaling. While SMAD7 inhibits both TGF-β and bone morphogenic protein-mediated SMAD signaling, SMAD6 is specific for bone morphogenic proteins. Studies by Massague and co-workers (16) have implicated SMAD7 in the effects of IFN-γ on the TGF-β mechanism. The mechanism by which IFN-γ blocks the TGF-β suppression of Muc4/SMC expression predicts expression of SMAD7 and formation of a SMAD7-TGF-β receptor complex, which is known to block SMAD2/3 activation. Based on these studies and others, we proposed that TGF-β can block Muc4/SMC synthesis via the SMAD pathway and that this repression can be reversed by IFN-γ via the Jak/STAT pathway and up-regulation of Smad7. Based on these studies, we analyzed the effectiveness of the SMAD7 antisense S-oligo to block SMAD7 protein expression.

To determine the requirement for SMAD7 in blocking the inhibitory effect of TGF-β on Muc4/SMC expression, cell cultures were treated with TGF-β and IFN-γ for a period of 24 h after the initial 24-h incubation with the S-oligo. This method required that both cytokines (TGF-β and IFN-γ) be present as opposed to the use of TGF-β alone in the SMAD2 antisense studies. If SMAD7 is required to block the TGF-β effect, we would expect reduced levels of Muc4/SMC protein expression in the presence of the SMAD7 antisense S-oligo. That is, the presence of TGF-β, as shown previously, would reduce the levels of Muc4/SMC protein expression, but if IFN-γ is simultaneously present, SMAD7 would be activated to block the effect of TGF-β. In our analyses we observed a 36% reduction in Muc4/SMC expression in SMAD7 antisense S-oligo-treated cell cultures compared with SMAD7 sense and scrambled S-oligo-treated cell cultures (Fig. 7B). The results indicate that TGF-β was able to inhibit Muc4/SMC expression in the presence of IFN-γ when SMAD7 expression was blocked by the antisense method. When SMAD7 expression was not blocked (sense and scrambled S-oligos), TGF-β suppression of Muc4/SMC expression was not observed (compared with SMAD7 antisense S-oligo-treated cell cultures). These data indicate a requirement for SMAD7 in regulating the post-translational effect of TGF-β on Muc4/SMC expression via the IFN-γ-Jak-STAT pathway.

DISCUSSION

Muc4/SMC is developmentally regulated in the rat mammary gland, dramatically increasing its levels of expression in the lactating mammary gland compared with the virgin mammary gland, while decreasing again during mammary gland involution (10, 11). Deviation from this regulatory mechanism...
can have adverse effects on the mammary gland, possibly propagating a cascade of deleterious events leading to neoplasia and tumor progression. Muc4/SMC has been shown to increase the potential for tumor growth (27) and metastasis (28), most likely due to its characteristic properties, such as anti-adhesion (4), anti-recognition (5), and modulation of cell signaling (6–8).

To elucidate the mechanism of action and factors that are involved in regulating expression of Muc4/SMC, we utilized primary cell cultures of rat mammary epithelial cells that are believed to mimic the in vivo state. Previously, TGF-β/H9252 was demonstrated to affect Muc4/SMC expression via a post-transcriptional mechanism (10). Additional studies have shown that TGF-β regulation of Muc4/SMC did not affect Muc4/SMC precursor synthesis or its rate of turnover. Instead, it was shown to interfere with the processing of Muc4/SMC precursor to its mature form of ASGP-1/ASGP-2 (11), suggesting a post-translational mechanism of action. In the current studies, we have furthered our understanding of TGF-β/H9252 regulation of Muc4/SMC by showing that SMAD2, and not SMAD3, is activated in MEC in response to TGF-β/H9252 (Fig. 4). SMAD2 was shown to translocate to the nucleus utilizing two methods: cell fractionation and immunocytochemistry. In addition, the requirement for SMAD2 activation in the regulation of Muc4/SMC expression was supported with the use of antisense technology (Fig. 6).

Various other growth factors and cytokines have been tested to determine their effects on Muc4/SMC expression. In addition to TGF-β, IFN-γ was shown to be involved in regulating Muc4/SMC expression. IFN-γ was observed to block the inhibitory effect of TGF-β on Muc4/SMC expression, and this effect was shown to be dose responsive (Fig. 2). A previous study by Ulloa et al. (16) integrated the TGF-β and IFN-γ signaling pathways. IFN-γ was shown to block TGF-β SMAD3 phosphorylation and activation by activating SMAD7 via the JAK-STAT-1 pathway.

To determine which STAT was activated in MEC upon IFN-γ stimulation, we screened STAT activation with monoclonal antibodies directed at STATs 1–6. IFN-γ is known to activate STATs 1, 3, 5a, and 5b (17, 21). It has been suggested that STAT-1 is the primary target of IFN-γ (21). STATs 2–6 were not activated in our experiments in MEC. However, STAT-1 was activated in response to IFN-γ (Fig. 5). This result suggested that STAT-1 plays a role in the regulation of Muc4/SMC expression. Additionally, SMAD7 was selected as a possible candidate in mediating the IFN-γ signal and regulating the TGF-β induced inhibition of Muc4/SMC expression (17, 19, 23, 24). Antisense inhibition assays revealed that SMAD7 was

![Fig. 7. Requirement for SMAD7 in the IFN-γ regulation of Muc4/SMC.](image_url)

![Fig. 8. Model for the proposed post-translational regulatory mechanism of Muc4/SMC expression by TGF-β and IFN-γ.](image_url)
required for blocking the effect of TGF-β on Muc4/SMC expression (Fig. 7).

The results of this study provide a clearer model of Muc4/SMC post-translational regulation by TGF-β and IFN-γ in the MEC (Fig. 8). Our model indicates that Muc4/SMC protein expression in MEC is inhibited post-translationally by the TGF-β pathway. Upon binding of TGF-β to its cognate receptor, SMAD2 is phosphorylated and activated by the TGF-β receptor via its intrinsic serine/threonine kinase activity. SMAD2 then forms a complex with co-SMAD4 and is translocated to the nucleus (14, 15) where it functions as a transcription factor to regulate gene expression of an unknown factor involved in Muc4/SMC precursor processing. The TGF-β-SMAD2 signaling pathway is blocked by activation of the IFN-γ Type II receptor, which functions to activate associated Jak tyrosine kinases that recruit STAT-1 by providing a docking site. STAT-1 serves as a substrate for Jak kinase activity. STAT-1 is activated, homodimerizes, and translocates to the nucleus to regulate transcription of the inhibitory SMAD7. SMAD7 then functions to block activation of SMAD2 and, therefore, blocks TGF-β signaling. The timing and concentration of the individual factors (TGF-β and IFN-γ) may determine the fate of Muc4/SMC expression.

This study raises other questions regarding the post-translational regulation of Muc4/SMC in the mammary gland by TGF-β and IFN-γ. An unknown factor is regulated by the TGF-β pathway that results in altering the processing of the precursor Muc4/SMC to its mature form. Processing of the Muc4/SMC precursor is known to involve a proteolytic cleavage, which in addition to glycosylation, results in the maturation of pMuc4/SMC to ASGP-1 and ASGP-2. Therefore, it is reasonable to suggest that TGF-β may negatively regulate an enzyme that either cleaves the pMuc4/SMC or is involved in modifying the cleavage site. Presently, a study is under way to investigate these possibilities. Preliminary experiments with various protease inhibitors seem to indicate the involvement of a proteolytic enzyme in this process. A better understanding of the mechanisms involved in regulating Muc4/SMC expression is key to determining the role of Muc4/SMC in other processes, particularly its role in tumor progression and metastasis.

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