Sialomucin Complex (Rat Muc4) Is Regulated by Transforming Growth Factor β in Mammary Gland by a Novel Post-translational Mechanism*

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Sialomucin complex (SMC, rat Muc4) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 (for ascites sialoglycoprotein-1) and a transmembrane subunit ASGP-2, produced from a single gene and precursor. SMC expression is tightly regulated in mammary gland; the level in lactating mammary gland is about 100-fold that in virgin gland. In rat mammary epithelial cells, SMC is post-transcriptionally regulated by Matrigel by inhibition of SMC precursor synthesis. SMC is also post-transcriptionally regulated by transforming growth factor-β (TGFβ). The repression of SMC expression by TGFβ is rapid, is independent of TGFβ-induced cell cycle arrest, and does not require new protein synthesis. Unlike Matrigel, TGFβ does not reduce SMC protein synthesis, as SMC precursor accumulation is equivalent in TGFβ-treated and untreated cells. Instead, SMC precursor in TGFβ-treated cells is more persistent and does not become processed as rapidly into mature ASGP-1 and ASGP-2, indicating that TGFβ disrupts processing of SMC precursor. These results indicate that SMC, a product of normal mammary gland and milk, is regulated by TGFβ by a novel post-transcriptional mechanism. Thus, SMC is regulated by multiple post-transcriptional mechanisms, which serve to repress potential deleterious effects of overexpression.

TGFβ is a member of a family of growth factors that have been shown to have extensive effects on the maturation and function of normal mammary gland. For example, TGFβ implants introduced into the mammary glands of subadult virgin mice can inhibit ductal development (1). In addition, overexpression of TGFβ1 in the mammary glands of transgenic mice inhibited lobuloalveolar development and milk protein production (2). TGFβ can induce expression of extracellular matrix proteins by human mammary epithelial cells in culture (3). Further, TGFβ can inhibit β-casein production by a post-transcriptional mechanism in mammary tissue explants from mid-pregnant mice (4, 5), although the molecular aspects of this mechanism are not presently known. Thus, in addition to its effects on mammary gland patterning, TGFβ appears to play a role in regulating accumulation of milk proteins during pregnancy.

TGFβ also regulates expression of another milk protein, SMC (6), which was originally discovered as a highly overexpressed glycoprotein complex on the surface of rat ascites 13762 mammary adenocarcinoma cells (7, 8). SMC consists of a peripheral O-glycosylated mucin subunit ASGP-1 (7–10) and an N-glycosylated integral membrane glycoprotein ASGP-2 (8, 11). The complex is transcribed from a single gene as a 9-kilobase pair transcript (12, 13) and translated into a single large polypeptide, which is proteolytically cleaved early in its biosynthesis. The subunits remain stably associated during transit to the cell surface (14). Recent studies have demonstrated that SMC is the rat homolog of human MUC4 (15). Cloning and sequencing of full-length human MUC4 showed 60–70% amino acid identities between human MUC4 and rat SMC in non-mucin regions of both the ASGP-1 and ASGP-2 (16, 17). MUC4 and SMC differ in their repeat domains in that the sequence of SMC does not contain the 16-amino acid repeat cloned and sequenced in the original description of MUC4 (17). The high degree of similarity between MUC4β, the human MUC4 analog of ASGP-2, and rat ASGP-2 provides strong evidence that they are homologous proteins. Several studies suggest that the two-subunit SMC is a multi-functional glycoprotein complex. Through its highly O-glycosylated tandem repeat domain, ASGP-1 can provide anti-recognition and anti-adhesive properties to tumor cells (9, 10, 18). Furthermore, SMC expression in tumor cells reduces their killing by natural killer cells (19). This anti-recognition property may be important to the high metastatic capacity of the 13762 ascites cells (7, 9, 20). ASGP-2 has two epidermal growth factor-like domains, which have all of the consensus residues present in active members of the epidermal growth factor family (12). Moreover, SMC has been shown to bind to and modulate phosphorylation of the receptor ErbB2 (21). Supporting the conclusion that ASGP-2 is a ligand is the observation that ErbB2 is constitutively phosphorylated in the 13762 ascites cells and associated with a multimeric complex of signaling components, including Src (22) and all of the components of the Ras to MAP kinase mitogenic pathway (23). Thus, the transmembrane subunit ASGP-2 is proposed to modulate signaling through the epidermal growth factor family of receptors via its interaction with ErbB2 (21, 24), the critical receptor for formation of active heterodimeric class I receptor tyrosine kinases (25). This interaction may play a role in the constitutive phosphorylation of ErbB2 in the 13762 ascites cells (22) and the rapid growth of these cells in vivo.

Sialomucin complex expression has been described in a number of normal secretory epithelial tissues in the adult rat (26, 27) and appears to have multiple and complex regulatory mech-
anisms. SMC protein is abundant in lactating mammary gland, but its level is very low in the virgin gland. However, the transcript for SMC is present at high levels in the virgin gland and does not change during pregnancy (6), suggesting that SMC expression is post-transcriptionally regulated in normal rat mammary gland. SMC synthesis is induced rapidly in cultured primary mammary epithelial cells from either normal pregnant or virgin rats. When mammary cells are cultured in Matrigel, a reconstituted basement membrane that stimulates casein expression, SMC protein, but not transcript levels, are significantly reduced. This post-transcriptional regulation is achieved by a ~10-fold reduction in SMC precursor biosynthesis when the cells are cultured in Matrigel. Interestingly, Matrigel has no effect on either the level of SMC or its transcript in cultured 13762 mammary tumor cells. TGFβ1 can also regulate SMC levels in normal cultured mammary epithelial cells, but not the asces tumors, by a post-transcriptional mechanism (6).

In the present study, we have characterized the mechanism of post-transcriptional regulation of SMC by TGFβ in cultured primary mammary epithelial cells. TGFβ inhibits induction of SMC expression when the cells are put into culture; the repression of SMC expression is rapid and is independent of TGFβ-induced cell cycle arrest. The presence of TGFβ does not affect the ratio of membrane-bound to soluble form of SMC produced, nor does it affect the rate of SMC turnover in these cells. Unlike Matrigel, which inhibits SMC precursor synthesis, TGFβ has little effect on SMC precursor synthesis. Instead, TGFβ alters the processing of SMC precursor into mature SMC (ASGP-2), a novel TGFβ action, which appears not to be a consequence of the effects of TGFβ on transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**—The MAT-B1 ascites subline of the 13762 rat mammary adenocarcinoma was maintained by weekly passage (28). Anti-ASGP-2 polyclonal antiserum was prepared against purified ASGP-2 (14) and has been used extensively for immunoprecipitations in previous studies (6, 14, 21, 26, 27). The mouse monoclonal antibody 4F12 was elicited using purified SMC, recognizes an epitope in the N-terminal 53 amino acids of ASGP-2 and has been used extensively for immunoprecipitations in previous studies (6, 21, 26, 27). Anti-C-Pep polyclonal antiserum used for immunoprecipitations was prepared against the C-terminal peptide of rat ASGP-2, N-SMNKFSYPDSEL-C (26). Anti-cyclin A polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Muc4 mouse monoclonal antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Cycloheximide was purchased from Calbiochem (La Jolla, CA). Puromycin and tunicamycin were purchased from Sigma. Cell culture medium was Ham’s F-12 medium supplemented with 10% FCS. After 24 h TGFβ was added to half the samples at a final concentration of 200 pM. After an additional 24 h, cells were washed twice with PBS, starved for 30 min in CyteMet-free Dulbecco’s minimal essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10 mM Heps, and incubated in 1 ml of labeling medium (starvation medium + 550 μCi/ml [35S]Cy/Cys + [35S]Met) (EXPRESS-SS Protein Labeling Mix, NEN Life Science Products) for times ranging from 0 to 6 h. For continuous labeling studies, labeled cells were washed twice with PBS and incubated with 100 μg/ml of 2% FCS in Ham’s F-12 medium supplemented with 10% FCS. After 24 h, cells were washed twice with PBS and lysed in 200 μl of 2% SDS. Lysed cells were boiled for 1 min, sonicated for 10 min in a bath sonicator, and diluted in 1 ml of Triton immunoprecipitation buffer (2.5% Triton X-100, 150 mM NaCl, 0.1% Triton X-100, 0.02% SDS, 5 mM EDTA, pH 7.4). Diluted cell lysates were centrifuged at 20,000 × g for 10 min at 4 °C. Cell lysates (equivalent counts used for samples for each time point) were immunoprecipitated with polyclonal anti-ASGP-2 antiserum and protein A-agarose beads (Sigma) overnight at 4 °C with rotation. Immunoprecipitates were washed with labeling wash buffer (50 mM Tris-buffered saline with 0.5% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Fc-specific; Pierce) diluted 1:20,000 in 1% bovine serum albumin-Tris-buffered saline with 0.5% Tween 20. Signals were detected with the Immobilon™ enhanced chemiluminescence kit (NEN Life Science Products).

**Labeling of Mammary Epithelial Cells**—Mammary epithelial cells were isolated from virgin rats and cultured on plastic in Ham’s F-12 medium supplemented with 10% FCS. After 24 h TGFβ was added to half the samples at a final concentration of 200 pM. After an additional 24 h, cells were washed twice with PBS, starved for 30 min in CyteMet-free Dulbecco’s minimal essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10 mM Heps, and incubated in 1 ml of labeling medium (starvation medium + 550 μCi/ml [35S]Cy/Cys + [35S]Met) (EXPRESS-SS Protein Labeling Mix, NEN Life Science Products) for times ranging from 0 to 6 h. For continuous labeling studies, labeled cells were washed twice with PBS and incubated in 200 μl of 2% SDS. Lysed cells were boiled for 1 min, sonicated for 10 min in a bath sonicator, and diluted in 1 ml of Triton immunoprecipitation buffer (2.5% Triton X-100, 150 mM NaCl, 0.1% Triton X-100, 0.02% SDS, 5 mM EDTA, pH 7.4). Diluted cell lysates were centrifuged at 20,000 × g for 10 min at 4 °C. Cell lysates (equivalent counts used for samples for each time point) were immunoprecipitated with polyclonal anti-ASGP-2 antiserum and protein A-agarose beads (Sigma) overnight at 4 °C with rotation. Immunoprecipitates were washed with labeling wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, 0.02% SDS, 5 mM EDTA, pH 7.4) six times for 10 min each at 4 °C with rotation. A fraction of immunoprecipitation supernatant was collected for analysis of total labeled protein. Washed immunoprecipitates were resuspended in 50 μl of SDS sample buffer, and the immunoprecipitate supernatant was diluted 1:1 in SDS sample buffer. Diluted samples (equivalent total counts per time point) were analyzed by SDS-PAGE and fluorography with Fluoro-Hance autoradiography enhancer (Research Products International Corp., Mount Prospect, IL).

**RESULTS**

**SMC (ASGP-2) Expression in Cultured MEC in the Presence or Absence of TGFβ**—We have previously shown that SMC/Muc4 protein is induced rapidly when isolated mammary epithelial cells are cultured on plastic tissue culture dishes. Further, we demonstrated that TGFβ post-transcriptionally regulates SMC in these cells. The aim of the current studies is to define the mechanism for post-transcriptional regulation of SMC by TGFβ. In all tissues studied to date, including mammary gland (8, 17), ASGP-1 and ASGP-2 are present as a complex, allowing us to use immunoblotting of ASGP-2 for the analysis of SMC. Moreover, our monoclonal antibody 4F12, which recognizes an epitope in the N-terminal 53 amino acids of ASGP-2, is more sensitive and more specific than those for ASGP-1, which were subsequently recognized with membrane-bound and soluble SMC (ASGP-2) and has been used extensively to study the expression of SMC (ASGP-2) in multiple tissues (26, 27).

A time course was performed to characterize the expression pattern of SMC (ASGP-2) in the presence or absence of TGFβ in cultured MEC. Isolated MEC from virgin rats were cultured on plastic in Ham’s F-12 medium supplemented with 10% fetal calf serum with or without 200 pM TGFβ. Cells were harvested at times ranging from 0 to 24 h after plating and lysed, and total protein was quantified. SMC (ASGP-2) content was analyzed by immunoblotting with mAb 4F12, and actin was measured by Lowry assay, and 5 μg of total protein was loaded for immunoblot analysis.

**Western Blotting**—For Western blots, SDS-PAGE was performed under reducing conditions using 6% polyacrylamide gels and the mini-Protean II system (Bio-Rad). Resolved proteins were transferred to nitrocellulose membranes and subsequently blocked in 5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20. After a 1-h incubation in primary antibody diluted in 1% bovine serum albumin-Tris-buffered saline with 0.5% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Fc-specific; Pierce) diluted 1:20,000 in 1% bovine serum albumin-Tris-buffered saline with 0.5% Tween 20. Signals were detected with the Immobilon™ enhanced chemiluminescence kit (NEN Life Science Products).
neutralizing antibody to TGFβ presence or absence of TGFβb cells cultured without TGFβb presence or absence of 200 pm TGFβ. Cells were harvested at the times indicated. Cell lysates were prepared for each time, and 5 µg of total protein were subjected to SDS-PAGE and immunoblot analysis with anti-ASGP-2 mAb 4F12 and anti-actin antibodies (A). B, quantitation of SMC (ASGP-2) expression by densitometric analysis of the bands from A.

The specificity of the TGFβ effect was studied by the addition of a neutralizing antibody to TGFβ. MEC were cultured on plastic in Ham’s F-12 medium supplemented with 10% fetal calf serum in the presence or absence of 200 pm TGFβ or a neutralizing antibody to TGFβ. 30 µl of anti-TGFβ antibody was incubated with the TGFβ for 30 min at 4 °C prior to addition to the culture. After 24 h the cells were analyzed for SMC (ASGP-2) by immunoblotting with mAb 4F12, and actin blotting was used as a loading control. In the presence of TGFβ, SMC (ASGP-2) levels were inhibited by approximately 50% (Fig. 2, A and B) as seen in Fig. 1A. However, in the presence of the neutralizing antibody, SMC (ASGP-2) levels were substantially less inhibited by TGFβ, indicating that the inhibition of SMC (ASGP-2) expression by TGFβ is specific. TGFβ is known to induce cell cycle arrest in epithelial cells, and the inhibition of SMC (ASGP-2) expression by TGFβ may be one of the outcomes of cell cycle arrest. To determine if inhibition of SMC (ASGP-2) expression is a result of reduced cell number by TGFβ treatment, MEC were cultured on plastic dishes in Ham’s F-12 supplemented with 10% fetal calf serum in the presence or absence of 200 pm TGFβ. After 24 h, cells were harvested using an enzyme-free cell dissociation buffer and counted. Cells were lysed, and equal numbers of cells or equal amounts of total protein were analyzed by immunoblot with mAb 4F12. The inhibition of SMC (ASGP-2) expression is apparent when equivalent cell numbers (Fig. 2C) or equivalent total protein is analyzed (Fig. 1A). These results indicate that reduction of SMC (ASGP-2) levels by TGFβ is not a result of reduction of cell number (or cell death).

To further investigate the relationship between SMC (ASGP-2) repression by TGFβ and the cell cycle, the timing of SMC (ASGP-2) repression by TGFβ was compared with that of TGFβ-induced cell cycle arrest. MEC from virgin rats were cultured on plastic dishes in Ham’s F-12 medium supplemented with 10% fetal calf serum in the presence or absence of 200 pm TGFβ. Cells were harvested after 24 or 48 h of culture for immunoblot analyses with mAb 4F12, anti-cyclin A, and anti-actin antibodies. During the first 24 h of culture, very little cyclin A is produced by the MEC, a marker for progression through the cell cycle (29), suggesting that the cells are not cycling (dividing) in the presence or absence of TGFβ (Fig. 3). However, during this time period, SMC (ASGP-2) levels are reduced in the TGFβ-treated cultures. During the second 24 h, cells cultured without TGFβ produce cyclin A, indicating that they are cycling. Those cells cultured with TGFβ produce less cyclin A, indicating that TGFβ is causing cell cycle arrest. However, the reduction in SMC (ASGP-2) levels in the TGFβ-treated cells are similar at the 24- and 48-h time periods. Thus, since TGFβ reduces SMC (ASGP-2) levels when MEC are not cycling, the reduction of SMC (ASGP-2) levels by TGFβ is independent of TGFβ-induced reduced cell cycle arrest. Moreover, these data suggest that reduction of SMC (ASGP-2) by TGFβ occurs by a different mechanism than TGFβ-induced cell cycle arrest. TGFβ can reduce SMC (ASGP-2) levels in cultured MEC in...
less than 24 h, suggesting that this is a rapid response. To determine more accurately how fast TGFβ can reduce SMC (ASGP-2) levels, MEC were cultured for 24 h to induce high levels of SMC (ASGP-2). TGFβ was then added to a final concentration of 200 pM to half of the cells, and samples were harvested 6 and 24 h later for immunoblot analyses. SMC (ASGP-2) expression was inhibited by TGFβ within 6 h of its addition (Fig. 4A); the inhibition was more pronounced 24 h after addition of TGFβ. The relatively rapid effects suggest that new transcription and protein synthesis may not be necessary for TGFβ-mediated repression of SMC (ASGP-2) levels. To test this idea, MEC from virgin rats were cultured for 24 h, then 200 pM TGFβ and/or 10 μg/ml cycloheximide were added to the media. Cells were harvested after 6 h for immunoblot analyses with mAb 4F12. As demonstrated previously, SMC (ASGP-2) levels were reduced by TGFβ within 6 h of its addition. The presence of cycloheximide, which inhibits new protein synthesis, did not reverse reduction of SMC (ASGP-2) levels by TGFβ (Fig. 4B), indicating that no new protein synthesis is required for TGFβ to reduce SMC (ASGP-2) levels.

**Effect of TGFβ on the Production of Soluble SMC (ASGP-2)—**

Normal mammary tissue produces both soluble and membrane forms of SMC (ASGP-2) in a ratio of ~60% membrane:40% soluble form (26). One possible effect of TGFβ is alteration of the ratio of membrane-bound to soluble form of SMC (ASGP-2) by stimulating conversion of the membrane precursor to soluble form. Thus, in the presence of TGFβ, the detectable SMC (ASGP-2) in the cell would be reduced because it would be secreted from the cell. To test this possibility, MEC were cultured in the presence or absence of 200 pM TGFβ for 48 h and lysed in radioimmunoprecipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris base, pH 8.0). The lysates were sequentially immunoprecipitated twice with anti-C-Pep, a polyclonal antibody that recognizes an epitope in the C-terminal (cytoplasmic) domain of SMC (ASGP-2), and once with polyclonal anti-ASGP-2. Two rounds of immunoprecipitation with anti-C-Pep will clear the cell lysate of membrane-bound form of SMC (ASGP-2) (26), while the polyclonal anti-ASGP-2 recognizes the remaining SMC (ASGP-2), the soluble form. This technique (with these antibodies) has been used to study the ratio of membrane-bound to soluble of SMC (ASGP-2) in multiple tissues (26, 27). Immunoprecipitates were analyzed by immunoblotting with mAb 4F12, which recognizes both membrane and soluble SMC (ASGP-2) (26). The presence of TGFβ does not affect the ratio of membrane to soluble form (Fig. 5A). Both treated and untreated cells produce ~55% membrane-bound and ~45% soluble form (Fig. 5B), and SMC (ASGP-2) soluble form was detected in the conditioned media from both treatment groups. The only difference was that the overall level of SMC (ASGP-2) produced in the TGFβ-treated cells was lower than that produced in untreated cells. These data rule out the possibility that the apparent decrease in SMC (ASGP-2) levels in the cultured MEC is due to a shift of membrane SMC (ASGP-2) to the soluble form.

**Effect of TGFβ on Turnover of SMC (ASGP-2)—**

Since the effect of TGFβ on SMC (ASGP-2) expression is rapid, another potential mechanism for its repression is the acceleration of SMC (ASGP-2) turnover. To investigate this possibility, virgin MEC cultured in the presence or absence of 200 pM TGFβ were treated with 5 μg/ml (final concentration) of cycloheximide or puromycin to inhibit new protein synthesis. Alternatively, MEC cultured in the presence or absence of TGFβ were treated with 5 μg/ml tunicamycin, a drug that inhibits N-glycosylation (30). We have found that treatment of MEC with tunicamycin inhibits new synthesis of SMC (ASGP-2), and as a result, this drug can be used as an alternative (potentially less toxic) method for inhibiting SMC (ASGP-2) synthesis. Cells were harvested at times ranging from 0 to 24 h after addition of inhibitors. Protein concentrations were quantified by Lowry assay, and 5 μg of total protein were subjected to immunoblot analysis with mAb 4F12. The stained bands were quantified by densitometry and the half-life of SMC (ASGP-2) in treated and untreated cells was estimated. Table I summarizes the estimated half-life of SMC (ASGP-2) in TGFβ-treated and untreated MEC for each inhibitor used. Thus, these data suggest that TGFβ does not significantly change the turnover of SMC (ASGP-2) in normal cultured MEC.

**Biosynthesis of SMC (ASGP-2) in the Presence or Absence of TGFβ—**

To investigate the effect of TGFβ on SMC (ASGP-2) translation, a labeling experiment was performed. MEC were cultured for 24 h, at which time half the cells were treated with 200 pM TGFβ. After an additional 24 h, the cells were labeled for times ranging from 0 to 6 h with [35S]Cys+[35S]Met. Cells were harvested, lysed, and immunoprecipitated with anti-ASGP-2 polyclonal antibody, which recognizes both the SMC

*S. A. Price-Schiavi, X. Zhu, R. Aquillin, and K. L. Carraway, unpublished observation."
Since TGF-β cultured in the presence or absence of TGF-β with mAb 4F12, anti-C-Pep. Immunoprecipitates were subjected to immunoblot analysis lysate was then immunoprecipitated with polyclonal anti-ASGP-2 to sequentially with anti-C-Pep to clear the lysate of membrane form. The value of the polyclonal anti-ASGP-2 band was divided by the total value to obtain the soluble form percentage. Values of both anti-C-Pep bands were added and divided by the total measurement was performed. MEC were cultured 24 h, and TGF-β was added to half the samples. After an additional 24 h, cells were metabolically labeled with [35S]Cys + [35S]Met. Following the pulse, the cells were washed in prelabeling medium twice and incubated in chase medium for times ranging from 1 to 8 h. TGF-β was present in half the samples at a concentration of 200 pM throughout the labeling procedure. After the chase, cell lysates were immunoprecipitated with anti-ASGP-2 antibodies. Immunoprecipitates as well as an aliquot of non-immunoprecipitated cell lysate were subjected to SDS-PAGE and fluorography. Total labeled protein was similar for both samples with and without TGF-β, suggesting that protein synthesis is not inhibited by TGF-β in these cells (Fig. 7A). The level of SMC (ASGP-2) precursor is similar for treated and untreated samples, again suggesting that TGF-β does not inhibit the translation of SMC (ASGP-2) (Fig. 7A). To determine whether TGF-β affects processing of SMC precursor into mature SMC (ASGP-2), the bands for SMC precursor and mature ASGP-2 were quantified by densitometry, and the results were plotted (Fig. 7, B and C). In the absence of TGF-β, SMC precursor is processed more slowly; after 4 h, only about 50% of SMC precursor had disappeared. In addition, much less mature ASGP-2 accumulated in the TGF-β-treated samples (Fig. 7C). The fact that ASGP-2 appears more slowly than precursor disappears suggests that unprocessed precursor is being degraded. These results indicate that TGF-β affects the processing of the SMC precursor into mature SMC (ASGP-2), causing the apparent reduction in SMC (ASGP-2) levels when cells are cultured in the presence of TGF-β. Once again, these

**TABLE I**

| Time Point | No TGF-β | 200 pM TGF-β |
|------------|----------|--------------|
| 0          | 4 h      | 4 h          |
| 1          | 12 h     | 12 h         |

Effect of TGF-β on Processing of SMC (ASGP-2) Precursor—Since TGF-β does not affect SMC translation or the turnover of the mature protein, another possibility is that TGF-β could affect the processing of the SMC precursor into mature ASGP-1/ASGP-2. In order to test this possibility, a pulse-chase experiment was performed. MEC were cultured 24 h, and TGF-β was added to half the cells to a final concentration of 200 pM. After an additional 24 h, the cells were pulse-labeled for 30 min with [35S]Cys + [35S]Met. Following the pulse, the cells were washed in prelabeling medium twice and incubated in chase medium for times ranging from 1 to 8 h. TGF-β was present in half the samples at a concentration of 200 pM throughout the labeling procedure. After the chase, cell lysates were immunoprecipitated with anti-ASGP-2 antibodies. Immunoprecipitates as well as an aliquot of non-immunoprecipitated cell lysate were subjected to SDS-PAGE and fluorography. Total labeled protein was similar for both samples with and without TGF-β, suggesting that protein synthesis is not inhibited by TGF-β in these cells (Fig. 7A). The level of SMC (ASGP-2) precursor is similar for treated and untreated samples, again suggesting that TGF-β does not inhibit the translation of SMC (ASGP-2) (Fig. 7A). To determine whether TGF-β affects processing of SMC precursor into mature SMC (ASGP-2), the bands for SMC precursor and mature ASGP-2 were quantified by densitometry, and the results were plotted (Fig. 7, B and C). In the absence of TGF-β, SMC precursor is processed more slowly; after 4 h, only about 50% of SMC precursor had disappeared. In addition, much less mature ASGP-2 accumulated in the TGF-β-treated samples (Fig. 7C). The fact that ASGP-2 appears more slowly than precursor disappears suggests that unprocessed precursor is being degraded. These results indicate that TGF-β affects the processing of the SMC precursor into mature SMC (ASGP-2), causing the apparent reduction in SMC (ASGP-2) levels when cells are cultured in the presence of TGF-β. Once again, these
mechanisms has evolved, involving responses at several levels. Indeed, we are just beginning to elucidate factors and mechanisms involved in regulation of this protein in mammary epithelial cells. Here, we demonstrate the mechanism by which SMC (ASGP-2) is regulated in mammary epithelia by TGFβ and provide evidence that this mechanism is different from that reported for regulation of SMC (ASGP-2) by Matrigel, a reconstituted basement membrane mimicking one type of extracellular matrix effect on the epithelium.

TGFβ has numerous effects on the normal developing mammary gland. It inhibits the growth of primary mammary epithelial cells as well as that of several transformed mammary epithelial cell lines (31–33). TGFβ can inhibit ductal growth in the virgin mouse mammary gland but does not influence alveolar morphogenesis or DNA synthesis in the alveolar cells of pregnant mice. These data suggest that TGFβ plays an important role in normal mammary gland patterning by controlling spacing of ducts to allow room for alveolar development during pregnancy, but does not affect alveolar development directly. In addition, TGFβ can inhibit casein and SMC (ASGP-2) synthesis in pregnant mouse mammary organ explant cultures (4) and isolated virgin or mid-pregnant (data not shown) MEC (6), respectively. On the other hand, Sudlow and others (5) report that TGFβ does not inhibit casein synthesis from lactating organ explant cultures or MEC from lactating mice. Taken together, these data suggest that TGFβ controls synthesis and accumulation of milk proteins during pregnancy in addition to its role in development.

We had shown previously that SMC (ASGP-2) levels could be regulated post-transcriptionally in cultured rat mammary epithelial cells by both Matrigel and TGFβ. In Matrigel regulation of the expression of SMC (ASGP-2) is markedly different from that of β-casein. Matrigel lowers SMC (ASGP-2) levels while it enhances β-casein levels. However, regulation of SMC (ASGP-2) and β-casein by TGFβ is similar. 1) Expression of both is repressed under conditions that do not inhibit total protein synthesis. 2) Both SMC (ASGP-2) and caseins are strongly inhibited by physiological picomolar doses of TGFβ (4, 6). 3) The mechanism of regulation appears to be post-transcriptional for both proteins. These data support a role for TGFβ as an inhibitor of milk protein synthesis and accumulation in the virgin or pregnant mammary gland.

TGFβ represses SMC (ASGP-2) levels in mammary epithelial cells whether or not the mammary epithelial cells are cycling. This result suggests that TGFβ-induced cell cycle arrest and TGFβ repression of SMC (ASGP-2) levels occur by different mechanisms (different signaling pathways). Administration of TGFβ to the mammary glands of pregnant mice does not influence DNA synthesis of alveolar cells, the cells that produce caseins and SMC (ASGP-2) (milk proteins) (1, 26, 34). Taken together, these data indicate that the repression of SMC (ASGP-2) levels by TGFβ is independent of the cell cycle and is not a result of growth inhibition. The repression of SMC (ASGP-2) expression by TGFβ is not the result of an increase in the production of the soluble, secreted form of SMC (ASGP-2), inhibition of biosynthesis of the SMC precursor, or an increase in SMC (ASGP-2) turnover. Instead, TGFβ interferes with the processing of SMC precursor into mature ASGP-1/ASGP-2, a novel post-translational effect and mechanism (Fig. 8).

In the mammary gland there are several different post-transcriptional mechanisms for controlling (milk) protein expression, and the specifics of these mechanisms are beginning to be elucidated. For example, SMC (ASGP-2) is regulated by Matrigel by inhibition of its biosynthesis and TGFβ by disrupting SMC precursor processing. β-Casein mRNA is stabilized by the presence of prolactin (35), and its synthesis is inhibited by...
TGFβ (4, 5). Lactoferrin message is induced and stabilized by cell rounding (36). Whey acidic protein has an undefined post-transcriptional regulatory mechanism. When MEC are cultured on plastic or basement membrane, whey acidic protein message is transcribed, but requires formation of a hollow alveolar structure with a closed lumen for its synthesis and secretion (37).

TGFβ has been implicated in a number of post-transcriptional regulatory mechanisms. TGFβ can regulate gene expression post-transcriptionally by increasing or decreasing the stability of mRNAs. In osteoblast cell cultures TGFβ can inhibit collagenase 3 expression by accelerating the decay of its transcript (38). In vascular smooth muscle cells TGFβ can stabilize lysyl oxidase mRNA (39). Other mechanisms of post-transcriptional regulation by TGFβ have also been proposed. For example, TGFβ inhibits cdk4 translation in Mv1Lu lung epithelial cells; the CDK4 5'-untranslated region is involved in its translational regulation (40). In human prostate cancer cell lines, TGFβ induces higher secreted levels of collagenase MMP-2 by increasing the stability of the secreted 72-kDa proenzyme (41). TGFβ represses SMC (ASGP-2) levels by disrupting SMC precursor processing, suggesting that it actually regulates one of the factors necessary for SMC precursor processing. This effect is rapid and does not require new protein synthesis. Thus, this appears to be a different post-transcriptional regulatory mechanism from others reported for TGFβ. The results in this study, along with another recent study, provide a clearer picture of the regulation of SMC (ASGP-2) in normal developing mammary gland and allow us to update our model. Virgin rat mammary epithelial cells are primed for SMC (ASGP-2) processing and its subsequent behavior. Additional experiments are in progress to investigate these possibilities.

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Fig. 8. Model for repression of SMC (ASGP-2) expression in normal mammary epithelial cells by extracellular matrix and TGFβ.
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