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Introduction

Sialomucin complex (SMC) was originally discovered as the major glycoprotein complex on the surface of highly malignant, metastatic 13762 rat ascites mammary adenocarcinoma cells (Sherblom and Carraway, 1980). The complex consists of a peripheral, O-glycosylated mucin subunit ASGP-1 (Sherblom *et al.*, 1980a,b), and an N-glycosylated integral membrane glycoprotein ASGP-2 to which ASGP-1 is tightly, but non-covalently, bound (Sherblom and Carraway, 1980; Hull *et al.*, 1990). Recent studies have demonstrated that SMC is the rat homolog of human MUC4 (Moniaux *et al.*, 1999). Several studies suggest that the two-subunit SMC is a multi-functional glycoprotein complex. Overexpression of SMC can provide anti-recognition and anti-adhesive properties to tumor cells (Komatsu *et al.*, 1997). Furthermore, SMC expression in tumor cells reduces their killing by natural killer cells (Komatsu *et al.*, 1999). 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 (Sheng *et al.*, 1992). Moreover, SMC has been shown to bind to and modulate phosphorylation of the receptor ErbB2 (Carraway *et al.*, 1999). Thus, the transmembrane subunit ASGP-2 is proposed to modulate signaling through the epidermal growth factor family of receptors via its interaction with ErbB2 (Carraway *et al.*, 1999; Carraway *et al.*, 1992). This interaction may play a role in the constitutive phosphorylation of ErbB2 in the 13762 ascites cells (Juang *et al.*, 1996) 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 including mammary gland (Rossi *et al.*, 1996; McNeer *et al.*, 1997) and appears to have multiple and complex regulatory mechanisms. Because overexpression of SMC may lead to deleterious consequences, it is important to understand how expression of this protein is regulated as well as the consequences of its interaction with ErbB-2. Thus, we are characterizing regulation of SMC expression in normal mammary epithelial cells and 13762 mammary ascites tumor cells. Furthermore, we are characterizing the interactions between ASGP-2 and ErbB-2 and the possible consequences of this interaction. For this final report we describe the regulation of SMC by extracellular matrix, TGF β , and interferon gamma (IFN- γ) in cultured primary mammary epithelial cells and tumor cells. SMC is regulated post-transcriptionally by extracellular matrix by inhibition of SMC precursor synthesis. TGF β alters the processing of SMC precursor into mature SMC (ASGP-1/ASGP-2). Downregulation of SMC expression by TGF β can be blocked by addition of IFN- γ to the culture media, suggesting that the SMAD pathway may be involved in regulation of SMC by TGF β . In addition, we have investigated the interaction of ASGP-2 and ErbB-2 in normal rat mammary epithelial cells. Both SMC and ErbB-2 are developmentally regulated in normal rat mammary tissue, and the two proteins form a complex in virgin as well as lactating mammary tissue. Although SMC and ErbB-2 have similar expression patterns in developing mammary tissue, they have different regulatory mechanisms in cultured mammary epithelial cells. Thus, we have studied consequences of the ASGP-2/ErbB-2 interaction in stably transfected tetracycline regulated A375 and MCF-7 cell lines where ASGP-2 is overexpressed upon removal of tetracycline from the culture medium. These studies demonstrate that overexpression of SMC on tumor cells blocks the therapeutic antibody herceptin from binding to ErbB-2, suggesting that overexpression of SMC on tumor cells may be a mechanism for resistance to some therapeutic approaches.

This report summarizes the results of studies outlined in DAMD17-97-1-7151: Regulation of Sialomucin Complex Expression and Its Effect on HER Receptor Interaction. In these studies we have elucidated some of the mechanisms involved in regulation of sialomucin complex (SMC) expression in normal mammary epithelial cells and tumor cells as well as investigated the interaction of SMC with the growth hormone receptor ErbB-2 and its potential effects on cancer therapies. For a detailed discussion of regulatory mechanisms of SMC in normal mammary epithelial cells (Tasks 1-5 in Statement of Work), please see the following appended manuscripts/reprints:

Price-Schiavi, S. A., Carraway, C. A. C., Fregien, N. L., and Carraway, K. L. (1998) Post-transcriptional regulation of a milk membrane protein, the sialomucin complex, (Ascites sialoglycoprotein (ASGP)-1/ASGP-2, Rat Muc4), by transforming growth factor β . J. Biol. Chem. 273, 35288 – 35237

Price-Schiavi, S. A., Zhu, X., Aquinin, R., and Carraway, K. L. (2000) Sialomucin Complex (Rat Muc4) is regulated by transforming growth factor β in mammary gland by a novel post-translational mechanism. J Biol Chem. 275, 17800-7

Zhu, X., Price-Schiavi, S. A., and Carraway, K. L. (2000) Extracellular regulated kinase (ERK)-dependent regulation of sialomucin complex/Muc4 expression in mammary epithelial cells. Oncogene. Sep 7;19(38):4354-4361

Effect of interferon gamma on SMC expression in normal cultured rat mammary epithelial cells

Effect of IFN-gamma on inhibition of SMC levels by TGF β -We have previously reported that TGF β inhibits expression of SMC protein in normal cultured rat mammary epithelial cells. Two signaling pathways have been implicated in TGF β effects: the SMAD pathways (Hartsough and Mulder, 1997) and the MAP kinase pathway (Massague, 1998). Preliminary experiments with MAP kinase pathway inhibitors suggest that SMC (ASGP-2) regulation by TGF β does not involve the MAP kinase pathway (unpublished data). Massague et al. reported that TGF β effects can be blocked by interferon gamma by activation of Jak1 and Stat1, which in turn upregulate expression of SMAD 7, an inhibitory SMAD (Ulloa, L., et al., 1999). Furthermore, normal cultured mouse mammary epithelial cells can respond to IFN-gamma by activation of Jak2 and Stat3 (Lee, Y.J., and Streuli, C.H., 1999). Thus, normal virgin mammary epithelial cell cultures were treated with TGF β and/or IFN-gamma to determine if the Jak/Stat and/or SMAD pathways may be involved in regulation of SMC by TGF β . Normal virgin mammary epithelial cells were isolated and cultured on plastic dishes in Ham's F-12 medium supplemented with 10% FBS. After 24 hours the medium was replaced with Ham's F-12 supplemented with ITS (5 ug/ml insulin, transferrin, and sodium selenite), 200 pM TGF β , and/or 100 ng/ml IFN-gamma. After an additional 24 hours in culture, cells were harvested, and 5 μ g total protein was subject to immunoblot analysis with monoclonal antibodies directed against SMC and actin (as a loading control). As expected TGF β inhibited SMC levels by approximately 50% (Fig. 1). Interferon gamma treatment alone had little effect on SMC levels, but surprisingly, SMC levels in cells

treated with both IFN-gamma and TGF β were similar to those in untreated cells. This result suggests that interferon gamma treatment can interfere with the inhibitory effects of TGF β on SMC levels in normal rat mammary epithelial cells.

Dose responsiveness of IFN-gamma blockage of TGF β effects on SMC levels-To determine if the interferon effect is dose responsive, virgin mammary epithelial cells were cultured as described above. During treatment with interferon gamma and TGF β , TGF β was added at 200 pM and interferon gamma was added at 0, 50, 100, and 200 ng/ml final concentration. After 24 hours of culture in these conditions, cells were harvested lysed, and 5 μ g total protein were subjected to immunoblot analysis with monoclonal antibodies directed against SMC and actin. Again, TGF β inhibited SMC levels by roughly 50%, while IFN-gamma alone had relatively little effect on SMC levels (Fig. 2A and B). At 50 ng/ml IFN-gamma did not block inhibition of SMC levels by TGF β , but at 100 and 200 ng/ml doses, IFN-gamma was able to block the TGF β effect. These data suggest that blockage of the inhibitory effects of TGF β on SMC levels by interferon gamma is dose responsive and that the balance of IFN-gamma and TGF β signals may determine the ultimate downstream effect.

Timing of IFN-gamma and TGF β effects on SMC levels-Massague et al. reported that IFN-gamma upregulates the inhibitory SMAD7 which interacts with the TGF β receptor complex and thus blocks activation of SMAD3. The upregulation of SMAD7 is rapid, occurring within 30 minutes of addition of IFN-gamma, and persists for at least 8 hours (Ulloa, L., et al., 1999). To determine the timing of the IFN-gamma effect in our system, mammary epithelial cells were isolated and cultured on plastic dishes in Ham's F-12 medium supplemented with 10% FBS. After 24 hours either TGF β or IFN-gamma was added at 200 pM or 100 ng/ml, respectively. After an additional 24 hours, IFN-gamma was added to the TGF β treated cells and TGF β was added to the IFN-gamma treated cells at the above concentrations and incubated for 2, 6, 12, or 24 hours. Cells were harvested, lysed and subjected to immunoblot analysis with anti-SMC and actin antibodies. When cells were treated with TGF β first, followed by IFN-gamma, SMC levels remained similar to those in cells treated only with TGF β (Fig. 3A and B). When cells were treated with IFN-gamma first, followed by TGF β , SMC levels remained high, similar to those in untreated cells or those treated with IFN-gamma alone. These data suggest that signaling through the TGF β and IFN-gamma pathways is persistent such that once a signaling cascade was set up by one factor, signaling from the other factor can not interfere.

Other experiments and further directions-Attempts were made to elucidate the specific pathways involved in the TGF β and IFN-gamma signaling pathways in normal rat mammary epithelial cells. Cells were isolated and treated as described in the first experiment. Cell lysates were subjected to immunoblot analysis with antibodies directed against Jak2, Stat3, phospho-Stat3, SMAD6, and SMAD7. Preliminary evidence suggests that Jak2 and Stat3 are present in these cells, but it seems that only cells treated with both IFN-gamma and TGF β have phosphorylated Stat3. These experiments need to be repeated for confirmation and further optimization of antibody conditions. Immunoblots performed with anti-SMAD6 and anti-SMAD7 antibodies were unsuccessful. Thus, the question remains whether an inhibitory SMAD is involved in blockage of TGF β effects by IFN-gamma. Again, optimization of antibody conditions

will need to be done before this question can be answered. Other issues that should be investigated are the contributions of these and other Jak/Stat and SMAD pathway members, when and where these contributions and interactions take place in the cell, and what effect IFN-gamma has on SMC levels in 13762 ascites tumor cells. These investigations could lead to further elucidation of the normal regulation of SMC in mammary gland as well as its disruption during tumor progression.

Interaction of SMC and ErbB-2 and its potential consequences

In order to characterize ASGP-2/ErbB-2 interactions in normal cultured mammary epithelial cells, it was necessary to try to establish culture conditions favorable for reasonable expression of both SMC (ASGP-2) and ErbB-2. Unfortunately, the expression patterns of SMC and ErbB-2 in normal cultured MEC are very different. SMC expression is highest when MEC are cultured on plastic in the presence of fetal calf serum, while culture of MEC in Matrigel substantially inhibits SMC levels (Price-Schiavi *et al.*, 1998, see accompanying reprint). However, ErbB-2 is maximally expressed when MEC are cultured embedded in Matrigel (SMC levels are very low under these culture conditions), while it is almost undetectable when MEC are cultured on plastic where SMC levels are highest.

This discrepancy in the *in vitro* expression patterns of SMC and ErbB-2 brings some difficulty to the experiments outlined for Tasks 6 and 7 in the Statement of Work for characterizing the ASGP-2/ErbB-2 interactions and downstream consequences. Thus, the studies outlined in Tasks 6 and 7 were pursued in A375 human melanoma and MCF7 human breast carcinoma cells lines stably transfected with SMC behind a tetracycline regulatable promoter. These cell lines are convenient for these kinds of studies because SMC expression can be turned off or on by the addition or removal of tetracycline from the tissue culture medium. SMC functions have been described extensively in these cells (Komastu, M. *et al.*, 1997, Carraway, KL III, *et al.*, 1999, Komastu *et al.*, 1999, Komastu *et al.*, 2000). These studies demonstrate that overexpression of SMC can block cell-cell and cell-matrix interactions, protect tumor cells from killing by the immune system, and potentiate metastasis of tumor cells. Furthermore, when SMC expression is induced, SMC and ErbB-2 can form a complex in both of these cell lines (Carraway, KL III, *et al.*, 1999). These studies led us to ask the question whether overexpression of SMC could provide a mechanism of resistance to some tumor therapies like Herceptin, a humanized monoclonal antibody directed against the extracellular domain of ErbB-2.

We began our studies with the A375 human melanoma cells because they were more extensively characterized and easier to work with than the MCF7 breast carcinoma cells. To determine if overexpression of SMC has any effect on the expression levels of ErbB-2, A375 cells were cultured for 72 hours in the presence or absence of tetracycline to repress or induce (respectively) SMC expression. Cells were harvested, lysed, and 5 ug total cell protein was subjected to immunoblot analysis with anti-ASGP-2 and anti-ErbB-2 antibodies. As expected, SMC levels were undetectable in the presence of tetracycline, and in the absence of tetracycline, SMC levels were very high. ErbB-2 levels, on the other hand, were equivalent whether or not SMC was expressed (Fig. 4). Thus, overexpression of SMC in the A375 cells does not affect ErbB-2 levels.

To determine what effect overexpression of SMC may have on antibody binding to ErbB-2, A375 cells were cultured as described above to turn on or off expression of

SMC. Cells were harvested in enzyme free cell dissociation buffer and analyzed for ErbB-2 by flow cytometry. The antibodies used for this analysis were Neomarkers antibody 2 (at a 1:100 dilution) and Calbiochem antibody 5 (at a 1:100 dilution), both anti-ErbB-2 antibodies directed against the extracellular domain of ErbB-2. In the absence of SMC, Neomarker antibody 2 stained approximately 45% of the cells while Calbiochem stained approximately 80% of the cells (Fig. 5). When SMC was overexpressed Neomarker antibody 2 only stained approximately 15% of the cells, while Calbiochem antibody 5 only stained approximately 55% of the cells. This represents a 30 – 60% decrease in antibody binding to ErbB-2. Since we have demonstrated that overexpression of SMC has no effect on overall ErbB-2 levels in these cells, these data suggest that overexpression of SMC blocks antibody binding to ErbB-2.

An antibody titration was performed to investigate whether using more antibody would lead to more ErbB-2 staining in the presence or absence of SMC. A375 cells were cultured as described above and analyzed by flow cytometry with the Neomarkers (at 1:50, 1:100, and 1:500 dilutions) and Calbiochem (at 1:50 and 1:500) antibodies described above. With either antibody, the amount of ErbB-2 staining increased when more antibody was used for staining (Fig. 6). However, when SMC was overexpressed, ErbB-2 staining was always lower (by roughly 30-75%) than when SMC expression was turned off.

Overexpression of SMC blocks cell-cell and cell-matrix interactions by non-specific steric hindrance (Komatsu, M., et al., 1997). Part of this demonstration was that overexpression of SMC reduced antibody binding to intracellular adhesion molecule (ICAM), but when SMC was capped with anti-SMC antibodies, ICAM staining was similar to that when SMC expression was low (Komatsu, M., et al., 1997). We have already demonstrated that SMC and ErbB-2 form a complex in the transfected A375 cells (Carraway, KL III, et al., 1999). Thus, to determine what effect capping SMC has on ErbB-2 antibody binding, A375 cells were cultured as described for 75 hours. Cells were harvested in enzyme free cell dissociation buffer and incubated with polyclonal anti-ASGP-2 antibody or pre-immune serum for thirty minutes. Cells were then analyzed by flow cytometry as described above. As expected, when SMC expression is low capping has no effect on ErbB-2 staining (Fig. 7). However, when SMC is overexpressed, capping actually reduces antibody binding to ErbB-2. If SMC and ErbB-2 were not in a complex, then capping SMC should relieve the block in antibody binding to ErbB-2. However, capping SMC actually caused a greater block to anti-ErbB-2 antibody binding, consistent with the formation of a complex between SMC and ErbB-2.

These studies were designed to address the question of what effect SMC overexpression may have on antibody based tumor therapies like herceptin. Thus, A375 cells were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested in enzyme free cell dissociation buffer and subjected to flow cytometry analysis using the humanized anti-ErbB-2 antibody Herceptin. Three different antibody concentrations were used for this study – 100 ug/ml, 10 ug/ml, and 1 ug/ml final concentration. The highest concentration used here is the average patient serum concentration of herceptin after 16 weeks of herceptin therapy. The lowest concentration was chosen because this was the final concentration of the antibodies used for flow cytometry in the studies described above. When SMC levels were low, almost 100% of the cells stained positively with any of the antibody concentrations used (Fig. 8). When

SMC was overexpressed, almost 100% stained when 100 ug/ml or 10 ug/ml herceptin was used. However, when 1 ug/ml herceptin was used for the analysis, only about 50% of the cells stained when SMC was overexpressed. The final serum concentration of an antibody does not necessarily represent the final local concentration of the therapeutic antibody at the site of the tumor. Thus, these data suggest that overexpression of SMC can block therapeutic antibody binding, providing a mechanism for tumor resistance to these types of therapies.

The A375 cells are human melanoma cells, not breast cancer cells. Thus, to determine if overexpression of SMC blocks ErbB-2 antibody binding on breast tumor cells, stably transfected MCF7 cells were analyzed in a manner similar to that described for the A375 cells. To ascertain whether SMC overexpression affects ErbB-2 expression, transfected MCF7 cells were cultured in the presence or absence of tetracycline for 72 hours as described for the A375 cells. Cells were then lysed and 5 ug total protein were subjected to immunoblot analysis with anti-ASGP-2 monoclonal antibody 4F12 or anti-ErbB-2 monoclonal antibodies. As expected, in the presence of tetracycline, SMC levels are undetectable, but in the absence of tetracycline, SMC is induced to a very high level (Fig. 9). MCF7 cells express similar modest levels of ErbB-2 whether or not SMC is expressed. Thus, SMC overexpression does not affect overall ErbB-2 levels in these cells.

To determine what effect SMC overexpression has on anti-ErbB-2 antibody binding, MCF7 cells were cultured as described above. Cells were harvested in enzyme free cell dissociation buffer and analyzed by flow cytometry with Calbiochem anti-ErbB-2 antibody 5 (at a 1:100 dilution). When SMC levels are low, approximately 25% of the cells stained positively, but when SMC levels were high, only about 12% of the cells stained positively (Fig. 10). Although these staining levels are low, this still represents about a 50% decrease in ErbB-2 antibody binding. This suggests that overexpression of SMC on breast tumor cells can block ErbB-2 antibody binding.

Studies on the effect of SMC overexpression on ErbB-2 antibody binding in MCF7 cells are still in progress. These cells are slow growing and are more difficult to work with than the A375 cells. Thus, the basic antibody binding experiments should be repeated for confirmation. Additionally, the antibody titer, the capping, and most importantly, the herceptin binding studies still need to be performed on these cells.

Key research accomplishments to date:

1. SMC is developmentally regulated in normal rat mammary gland largely by a post-transcriptional mechanism.
2. Matrigel (reconstituted ECM) post-transcriptionally regulates SMC levels in normal rat MEC by inhibition of SMC precursor synthesis.
3. SMC levels in 13762 MAT-B1 tumor cells are unaffected by Matrigel.
4. SMC is post-translationally regulated in normal rat MEC by TGF β by disruption of SMC precursor processing. (Note that this is a different mechanism than that described for Matrigel.)
5. SMC expression is unaffected by TGF β in 13762 MAT-B1 tumor cells.
6. SMC and ErbB-2 have similar expression patterns in normal developing rat mammary gland.
7. SMC and ErbB-2 can form a complex in both virgin and lactating mammary gland.
8. SMC and ErbB-2 have different mechanisms of regulation in cultured normal rat MEC.
9. The inhibitory effect of TGF β on SMC expression can be blocked by IFN- γ in a time and dose dependent manner.
10. Overexpression of SMC on tumor cell surfaces can block antibody binding (including herceptin) to ErbB-2.

Reportable outcomes:

1. Papers/manuscripts:

Price-Schiavi, S. A., Carraway, C. A. C., Fregien, N. L., and Carraway, K. L. (1998) Post-transcriptional regulation of a milk membrane protein, the sialomucin complex, (Ascites sialoglycoprotein (ASGP)-1/ASGP-2, Rat Muc4), by transforming growth factor β . *J. Biol. Chem.* 273, 35288 – 35237

Carraway, K.L., Price-Schiavi, S.A., Zhu, X., and Komatsu, M. (1999) Regulation of expression of sialomucin complex (rat Muc4), the intramembrane ligand for ErbB2, at the transcriptional, translational and post-translational levels in rat mammary gland. *Cancer Control* 6, 613-614

Carraway, K.L., Price-Schiavi, S.A., Komatsu, M., Idris, N., perez, A., Li, P., Jepson, S., Zhu, X., Carvajal, M.E., and Carraway, C.A.C. (2000) Multiple facets of sialomucin complex/MUC4, a membrane mucin and ErbB-2 ligand, in tumors and tissues (Y2K update) *Frontiers in Bioscience* 5, 95-107

Price-Schiavi, S. A., Zhu, X., Aquinin, R., and Carraway, K. L. (2000) Sialomucin Complex (Rat Muc4) is regulated by transforming growth factor β in mammary gland by a novel post-translational mechanism. *J Biol Chem.* 275, 17800-7

Zhu, X., Price-Schiavi, S. A., and Carraway, K. L. (2000) Extracellular regulated kinase (ERK)-dependent regulation of sialomucin complex/Muc4 expression in mammary epithelial cells. *Oncogene*. In press

Price-Schiavi, S. A., Idris, N., Li, P., Carraway, C. A. C., and Carraway, K. L. Interaction of sialomucin complex (SMC, rat Muc4) with ErbB-2 in developing rat mammary gland and 13762 mammary tumor cells. Manuscript in preparation

Price-Schiavi, S. A., Falkenburg, R. V., Ramsauer, V., and Carraway, K. L. Interferon gamma (IFN- γ) blocks downregulation of sialomucin complex (SMC/Rat Muc4) expression in normal rat mammary epithelial cells. Manuscript in preparation

Price-Schiavi, S. A., Jepson, S., Li, P., Carvajal, M. E., and Carraway, K. L. Overexpression of sialomucin complex (SMC/rat Muc4) on tumor cell surfaces blocks antibody binding to ErbB-2. Manuscript in preparation.

2. Abstracts and presentations:

Post-transcriptional regulation of a milk membrane protein, Sialomucin complex, by TGF β

Price-Schiavi, S.A., Carraway, C.A.C., Fregien, N.L., and Carraway, K.L.
Poster presentation at American Society for Cell Biology, San Francisco CA, December, 1998

Post-transcriptional regulation of sialomucin complex in normal rat mammary gland by TGF β

Price-Schiavi, S.A., Fregien, N.L., Carraway, C.A.C., and Carraway, K.L.
Poster presentation at Nature BioTechnology Winter Symposium, Miami FL, February, 1999

Characterization of the TGF β effect on sialomucin complex (Rat MUC-4) expression in normal rat mammary epithelial cells

Price-Schiavi, S.A., Zhu, X., and Carraway, K.L.
Poster presentation at American Society for Biochemistry and Molecular Biology, San Francisco CA, May 1999

Mechanisms for post-transcriptional regulation of SMC expression in normal rat mammary epithelial cells

Price-Schiavi, S.A., Aquinin, R., and Carraway, K.L.
Poster presentation at Gordon Conference on Mammary Gland Biology, Henniker NH June 1999

3. Degrees obtained that were supported by award DAMD17-97-1-7151:

Shari A. Price-Schiavi, Doctor of Philosophy in Molecular Cell and Developmental Biology, University of Miami, December 1999

4. Employment received on experiences/training supported by DAMD17-97-1-7151:

Shari A. Price-Schiavi, Post-doctoral associate, Department of Cell Biology and Anatomy, University of Miami, laboratory of Dr. Kermit Carraway, January – June 2000

Shari A. Price-Schiavi, Post-doctoral associate, Sylvester Comprehensive Cancer Center University of Miami, laboratory of Kelvin Lee, M.D., June 2000 - present

Conclusion

The work summarized in this report describes the regulation of SMC in normal mammary epithelia and adds to our current understanding of its misregulation in the 13762 mammary tumor cells. SMC is developmentally regulated in normal rat mammary gland by a posttranscriptional mechanism. Although a number of factors have some effect on SMC levels in primary mammary epithelial cell cultures, Matrigel and TGF β can mimic the posttranscriptional regulation of SMC seen *in vivo*. Interestingly, the regulatory mechanisms for these two factors are different. Matrigel inhibits synthesis of SMC precursor, while TGF β interferes with SMC precursor processing. These data indicate both posttranscriptional and posttranslational mechanisms of regulation, which further illustrates the complexity of regulating mammary gene expression.

From these data, we can propose the following model. Virgin rat mammary epithelial cells are primed for SMC production by the presence of SMC transcript, whose expression is regulated by cell differentiation and insulin/insulin-like growth factor (Zhu, X *et al.*, 2000). Translation of this transcript is repressed by an inhibition related to cell environment (cell shape or context), mimicked by Matrigel. High levels of TGF β in the virgin mammary gland further control translated SMC precursor by regulating its processing. As pregnancy proceeds the cell environment changes, and active TGF β levels drop (Barcellos-Hoff, personal communication), allowing for increased translation and processing. Finally, at the onset of lactation TGF β levels become undetectable, and SMC is translated and processed to its full capacity. Disruption of the cell environment by cell isolation and loss of TGF β signaling causes the overexpression of SMC in MEC, which can be reversed by Matrigel and TGF β addition.

Loss of TGF β responsiveness is a significant factor in tumor progression, and may be involved in the overexpression of SMC in the MAT-B1 ascites tumor cells. Neoplastic transformation can lead to a loss of cell polarization and basement membrane contact, releasing the inhibition on precursor synthesis. The loss of TGF β responsiveness during tumor progression will release the post-translational processing block and lead to frank over-expression of SMC with its potential for deleterious consequences. Indeed, in the MAT-B1 tumor cells regulation by both Matrigel and TGF β appears to have been disrupted, and this, in combination with the 5-fold amplification of the gene and overexpression of the transcript contributes to its gross overexpression in this tumor cell line. The growth factor-like properties of SMC may then contribute to uncontrolled proliferation of these cells, while the mucin subunit contributes to protection from the immune system and loss of adhesion. In summary, SMC is an unusual post-transcriptionally regulated milk membrane protein, whose overexpression contributes properties conducive to tumor progression.

SMC and ErbB-2 are both developmentally regulated in the normal mammary gland. Although the two proteins have slightly different expression patterns and appear to have different regulatory mechanisms, the two proteins can form a complex in normal mammary epithelial cells as they do in the 13762 ascites tumor cells. Thus, a major question to be explored is what is the function of SMC itself and of the SMC/ErbB-2 complex in normal developing mammary gland? One way to address this question would be to study transgenic mice that overexpress SMC and/or ErbB-2 in the mammary gland and look for gross morphology and/or functional changes in the developing gland. These mammary cells could also be studied for more specific changes at the cellular level.

Another approach would be to knock out SMC and/or ErbB-2 in the mammary gland and look for morphology or functional changes in the developing gland. Again, these mammary epithelial cells could be studied for more specific changes at the cellular level. It would be of interest to see if there was any increase in the development of spontaneous mammary tumors in either case.

These studies raise other questions about regulation of SMC expression in normal mammary gland. What are the specifics of cell context that regulate SMC synthesis? These issues may include cell adhesion or cell shape and may involve the cytoskeleton. What is the signaling pathway TGF β uses to regulate SMC in mammary epithelial cells? There are two signaling pathways currently described for TGF β - the MAP kinase pathway (Hartsough and Mulder, 1997) and the SMAD associated pathways (Massague, 1998). Preliminary data from our lab suggest that SMC regulation by TGF β does not involve the MAP kinase pathway, although this does not necessarily mean that it involves the SMAD pathway. Furthermore, is this same signaling pathway used to regulate casein synthesis during pregnancy? Another issue is what SMC processing protein is TGF β affecting? Because the SMC precursor is more persistent in TGF β treated cells, the affected protein may be the enzyme that cleaves SMC precursor into ASGP-1 and ASGP-2. Other mucins have the same consensus sequence, N-GDPH-C, at their putative cleavage site (Carraway *et al.*, 1992), suggesting that they may be cleaved (processed) by the same enzyme (or family of enzymes). Thus, if the cleavage enzyme is regulated by TGF β , this mechanism of regulation may be applicable to other mucins. Elucidation of SMC's regulatory mechanisms by TGF β and other factors in normal developing mammary gland and its disruption in the 13762 tumor cells will give further insight into both normal developmental processes and tumor progression.

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cells by transforming growth factor β . Gordon Conference for Mammary Gland Biology
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Table 1. Effect of exogenous factors on SMC levels in cultured normal mammary epithelial cells

Factor	Change in SMC levels
Serum free media	Reference point
Fetal calf serum (10%)	↑↑↑↑↑
Fetal calf serum (1%)	↑↑↑
Insulin	↑↑
Hydrocortisone	↑↑
Prolactin	↓
Estrogen	-
Progesterone	-
EGF	-
NDF	-
TGF- β	↓↓↓
Collagen-I	↑
Collagen-IV	↑
Fibronectin	↑
Laminin	↑
Vitronectin	↑
Matrigel	↓↓↓

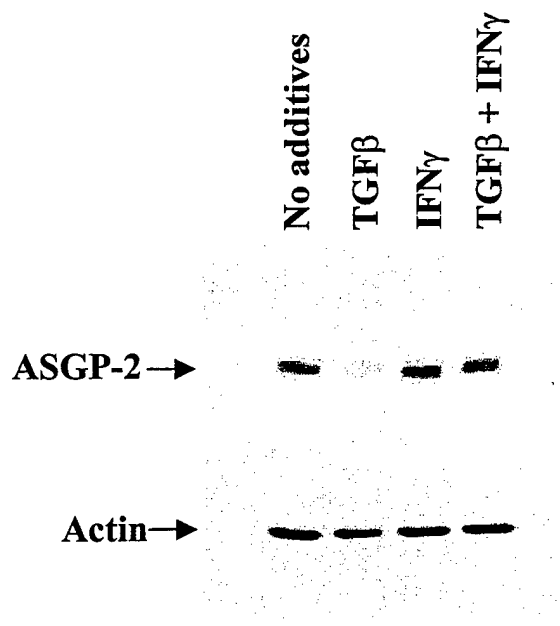


Figure 1. Effect of IFN-gamma on inhibition of SMC levels by TGFβ in normal rat mammary epithelial cells. Normal virgin rat mammary epithelial cells were isolated and cultured on plastic dishes in Ham's F-12 medium supplemented with 10% FBS. After 24 hours the medium was replaced with serum free medium supplemented with insulin, transferrin, sodium selenite, TGFβ (200 pM) and/or IFN-gamma (100 ng/ ml). After an additional 24 hours in culture, cells were harvested, and 5 ug total protein was analyzed by immunoblot with anti-ASGP-2 monoclonal antibody 4F12 or anti-actin mmonoclonal antibody as indicated in the figure.

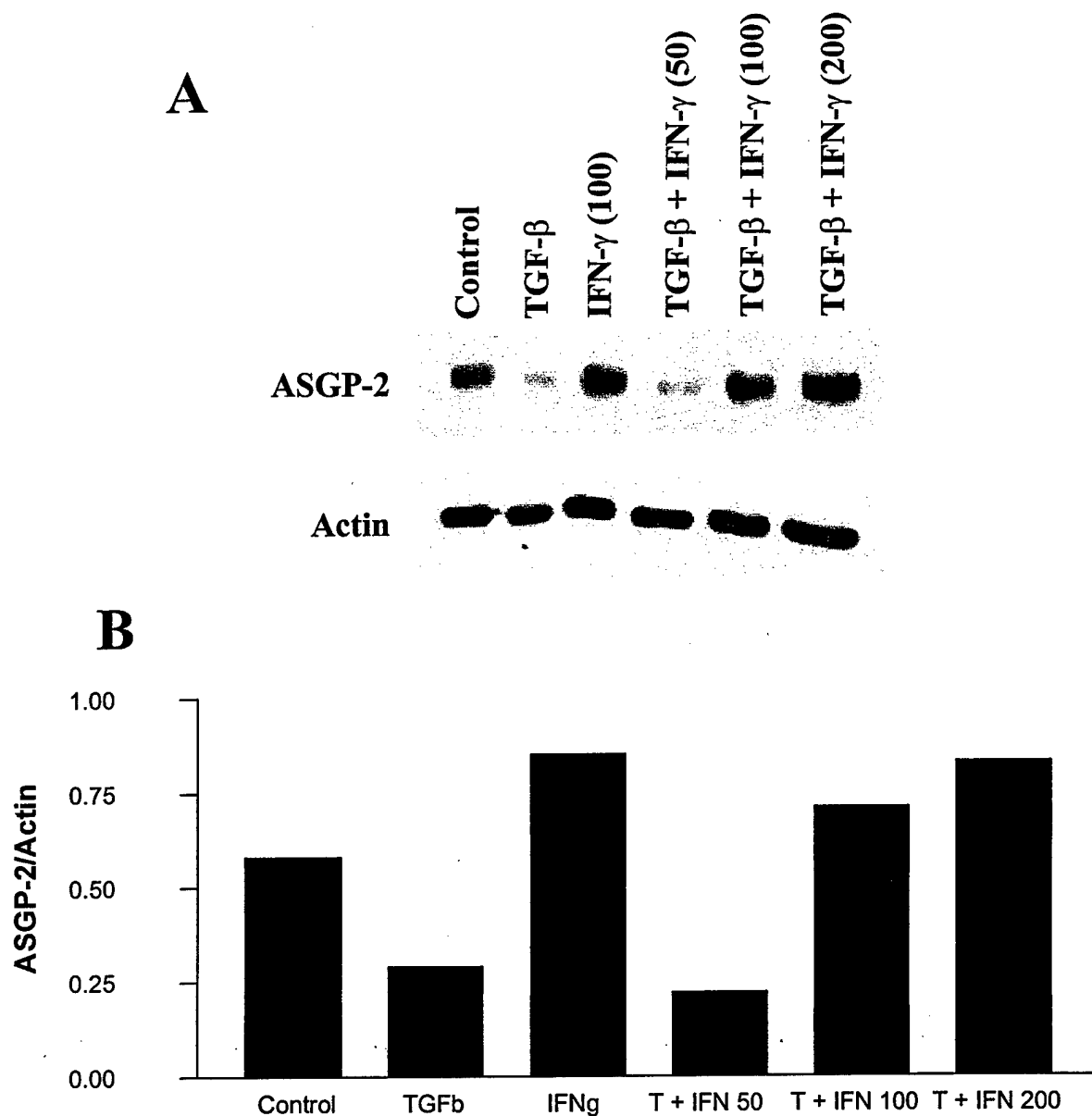


Figure 2. Dose responsiveness of IFN-gamma effect in normal mammary epithelial cells. Normal virgin mammary epithelial cells were isolated and cultured on plastic dishes in Ham's F-12 medium supplemented with 10% FBS. After 24 hours the medium was replaced with serum free medium containing insulin, transferrin, sodium selenite, TGF β , and/or IFN-gamma at 0, 50, 100, or 200 ng/ml as indicated at the top of the figure. A) Immunoblot analysis of TGF β and IFN-gamma treated cells. Cell lysates were subjected to immunoblot analysis with anti-ASGP-2 monoclonal antibody 4F12 or anti-actin monoclonal antibodies. B) Normalized graphical representation of the data in A). The ASGP-2 and actin bands in A) were quantified by densitometry. The value of the ASGP-2 band was divided by the value of the actin band and the results were plotted.

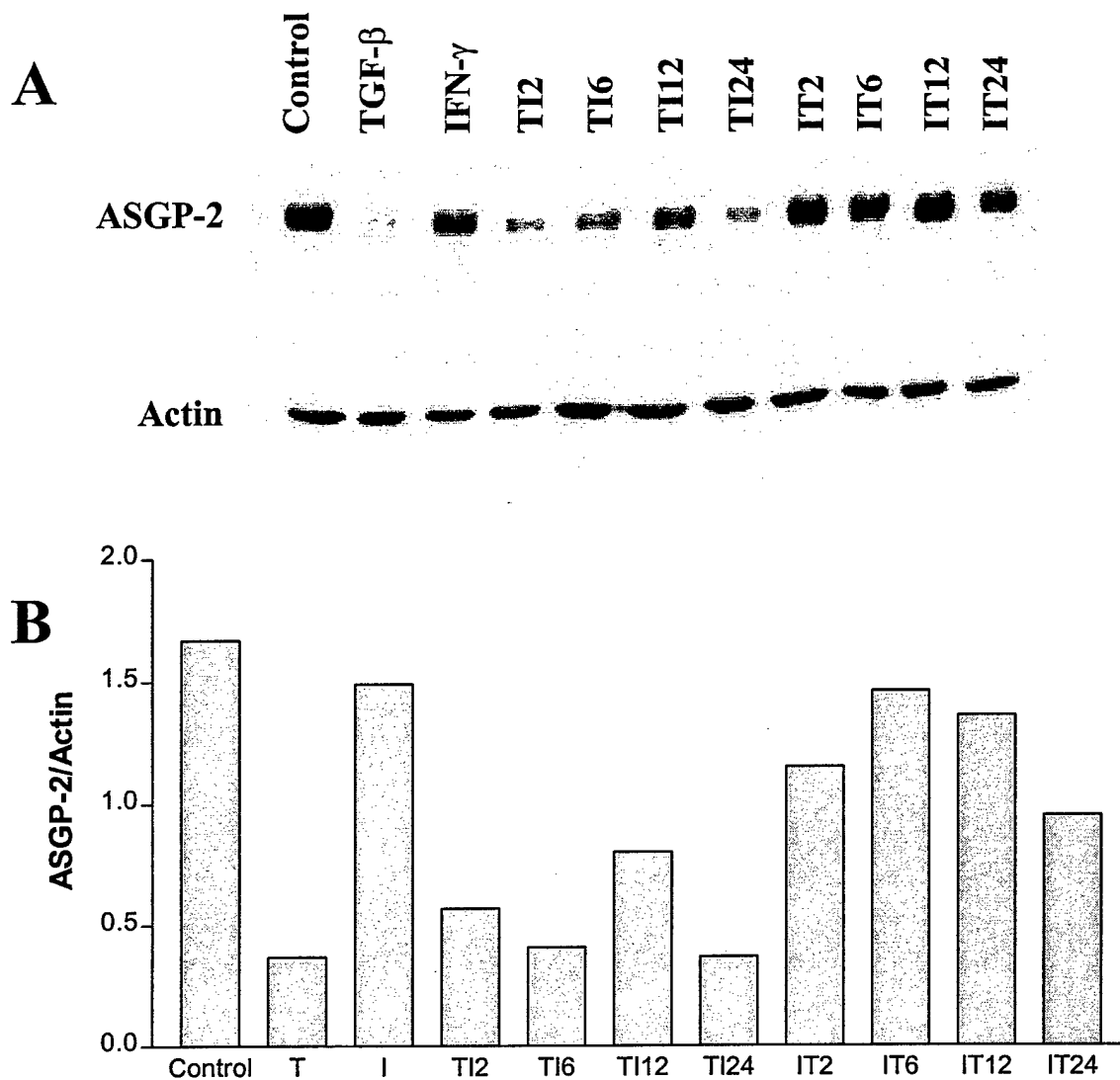


Figure 3. Timing of the effect of IFN-gamma on inhibition of SMC levels by TGF β . Normal virgin mammary epithelial cells were cultured in Ham's F-12 medium supplemented with 10% FBS. After 24 hours, the medium was replaced with serum free medium supplemented with insulin, transferrin, sodiumselenite, 200 pM TGF β , or 100 ng/ml IFN-gamma. After another 24 hours IFN-gamma (100 ng/ml) was added to the TGF β treated samples, and TGF β was added to the IFN-gamma treated samples. Cells were incubated under these conditions for the times indicated at the top of the figure. A) Immunoblot of TGF β and IFN-gamma treated cells. Cell lysates were subjected to immunoblot analysis with anti-ASGP-2 monoclonal antibody 4F12 and anti-actin monoclonal antibodies as indicated at the left of the figure. B). Normalized graphical representation of the data in A). The ASGP-2 and actin bands in A) were quantified by densitometry. The value of the ASGP-2 band was divided by the value of the respective actin band and the results were plotted. Abbreviations: T: TGF β , I: IFN-gamma, TI: TGF β first followed by IFN-gamma, IT: IFN-gamma first followed by TGF β .

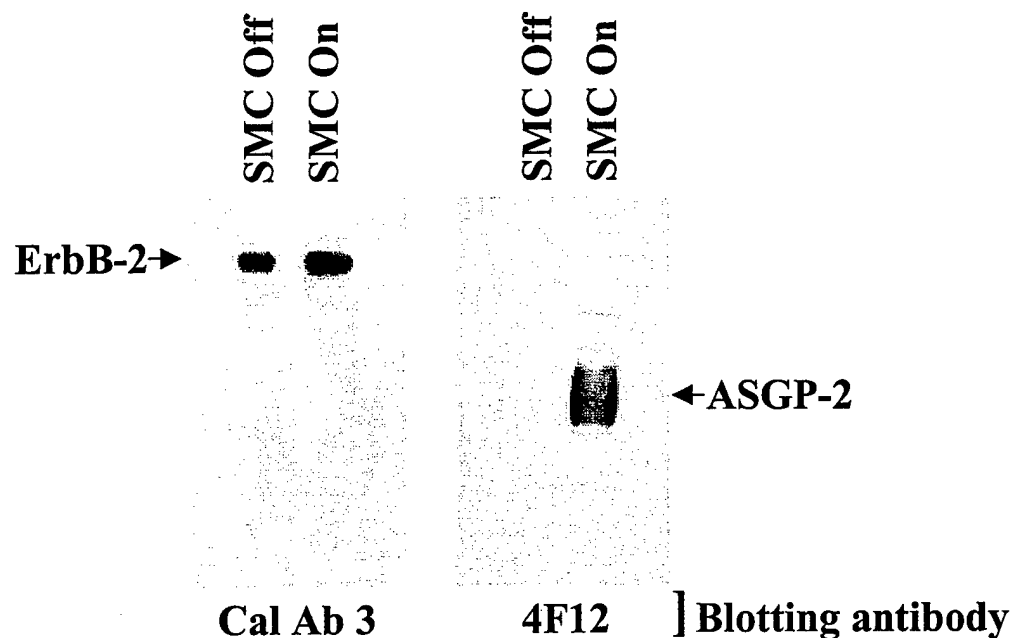


Figure 4. Effect of SMC expression on ErbB-2 levels in A375 cells. A375 cells stably transfected with tetracycline regulatable SMC were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested, lysed, and 5 ug total protein were analyzed by immunoblot with anti-ErbB-2 and anti-ASGP-2 monoclonal antibodies. Antibodies used for the blots were anti-ErbB-2 Calbiochem antibody 3 and anti-ASGP-2 monoclonal antibody 4F' 12.

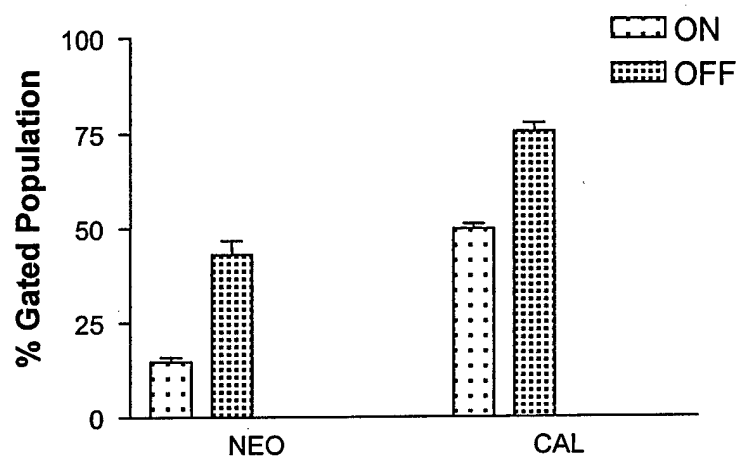


Figure 5. Effect of SMC expression on ErbB-2 antibody binding in A375 cells. A375 cells stably transfected with tetracycline regulatable SMC were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested and stained with anti-ErbB-2 antibodies Neomarkers antibody 2 (Neo) and Calbiochem antibody 5 (Cal) and analyzed by flow cytometry. The antibody used for each staining is indicated at the bottom of the graph.

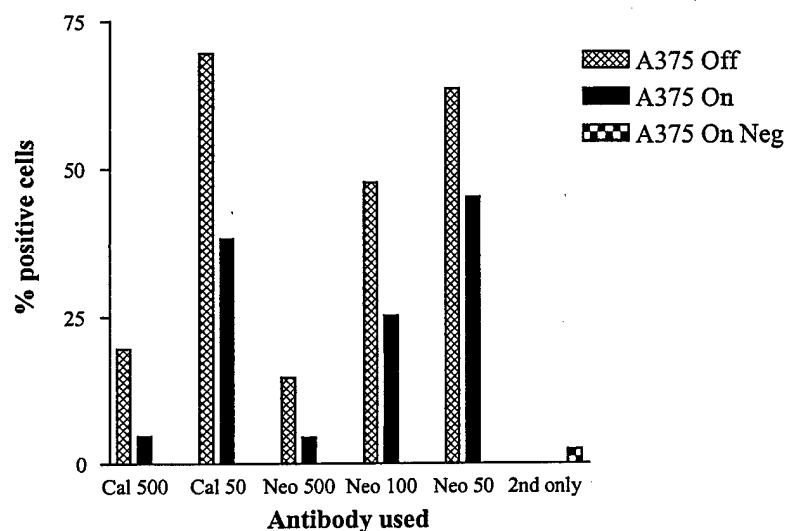


Figure 6. Effect of SMC expression on anti-ErbB-2 antibody binding using increasing concentrations of ErbB-2 antibodies in A375 cells. A375 cells stably transfected with tetracycline regulatable SMC were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested and subjected to flow cytometry using anti-ErbB-2 antibodies Calbiochem antibody 5 (Cal) at 1:500 and 1:50 dilutions and Neomarkers antibody 2 at 1:500, 1:100, and 1:50 dilutions as indicated at the bottom of the graph.

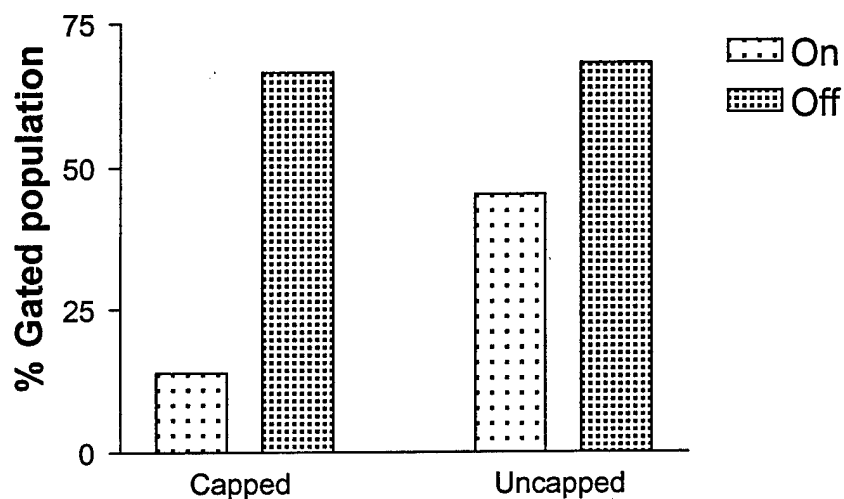


Figure 7. Effect of capping SMC on anti-ErbB-2 antibody binding in A375 cells. A375 cells were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested and incubated with either polyclonal anti-ASGP-2 antibody or preimmune serum for 39 minutes. Cells were then stained with anti-ErbB-2 Calbiochem antibody 5 and analyzed by flow cytometry.

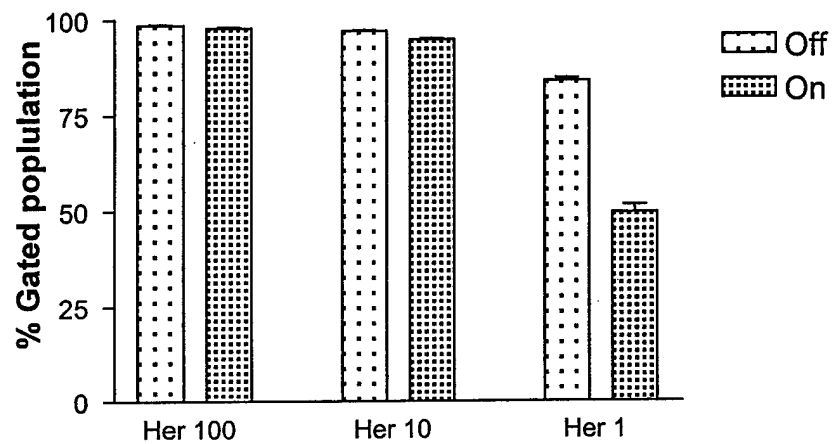


Figure 8. Effect of SMC expression on Herceptin binding in A375 cells. A375 human melanoma cells stably transfected with SMC under a tetracycline regulatable promoter were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested in enzyme free cell dissociation buffer and analyzed by FACS using Herceptin, a humanized monoclonal antibody against the extracellular domain of ErbB-2.

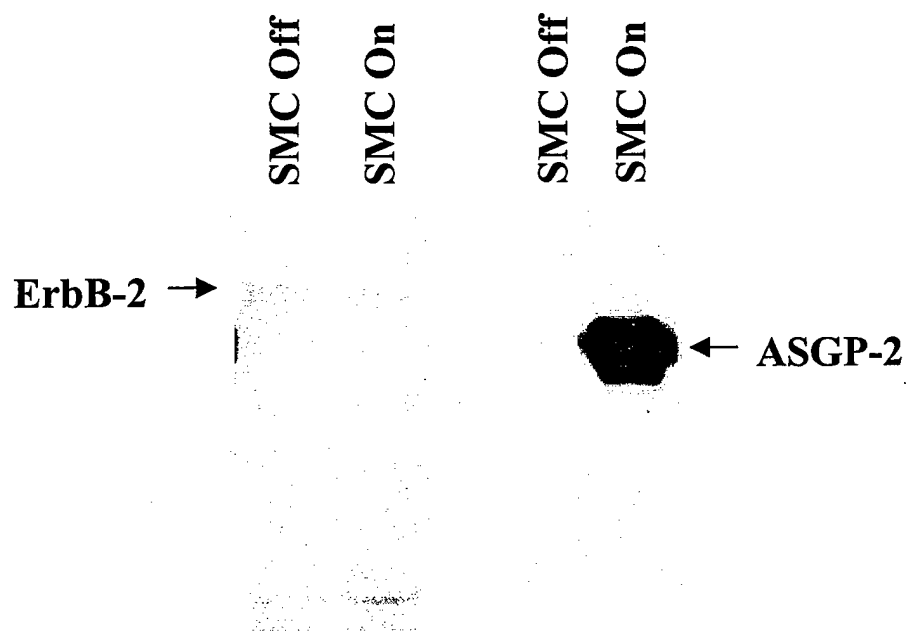


Figure 9. Effect of SMC expression on ErbB-2 levels in MCF-7 human breast cancer cells. MCF-7 cells stably transfected with SMC under a tetracycline regulatable promoter were cultured in the presence or absence of tetracycline for 72 hours. Cells expressing or not expressing SMC were harvested, lysed and subjected to immunoblot analysis with anti-ErbB-2 and anti-ASGP-2 monoclonal antibodies as indicated at the sides of the blots.

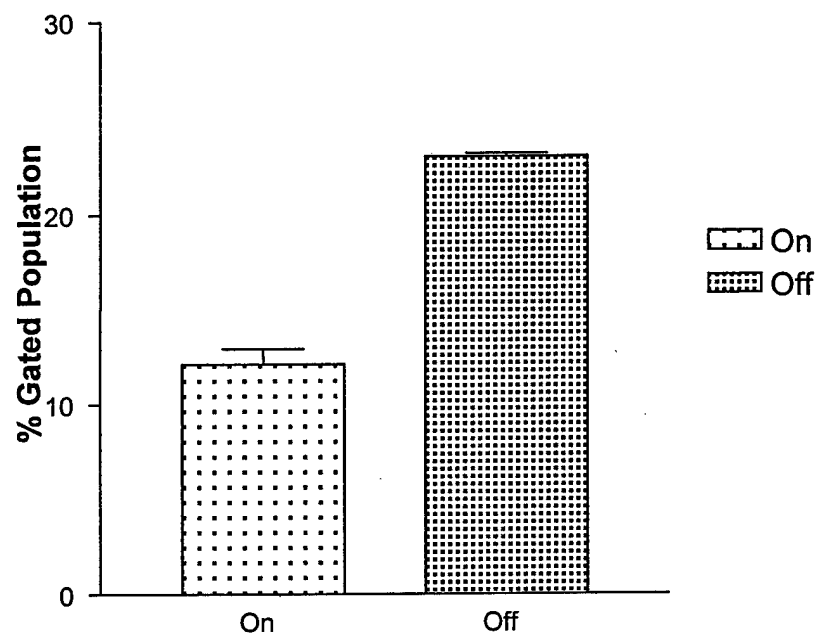


Figure 10. Effect of SMC expression on ErbB-2 antibody binding in MCF7 cells. MCF7 cells stably transfected with tetracycline regulatable SMC were cultured in the presence or absence of tetracycline for 72 hours. Cells were harvested and stained with anti-ErbB-2 Calbiochem antibody 5 at a 1:100 dilution and analyzed by flow cytometry.

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Extracellular Regulated Kinase (ERK)-dependent Regulation of Sialomucin Complex/rat Muc4 in Mammary Epithelial Cells

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ERK regulation of SMC/Muc4 in mammary epithelial cells

Key words: Muc4, ERK, ErbB2, mammary, epithelial, transcript regulation

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FOOTNOTE

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SUMMARY

Sialomucin complex (SMC, rat Muc4) is a membrane mucin implicated in the protection of epithelia and the metastasis of some tumors. It is a heterodimeric complex, containing a mucin subunit with anti-adhesive activity and a transmembrane subunit with epidermal growth factor-like domains, one of which acts as an intramembrane ligand for ErbB2. Serum, insulin and insulin-like growth factor, but not epidermal growth factor, induce the expression of sialomucin complex in mammary epithelial cells. Induction correlates with sustained, but not transient, activation of extracellular-regulated protein kinase (ERK). MEK inhibitor U0126 blocked the induction, while activated MEK-1 transfected into a rat mammary adenocarcinoma cell line induced a sustained activation of ERK and up-regulated SMC/Muc4 expression. Northern and Western blotting indicated that up-regulation occurred concomitantly at the transcript and protein levels, both of which could be blocked by U0126. These results suggest that expression of SMC/Muc4 in mammary epithelial cells is regulated by selected growth factors through an ERK-dependent pathway at the transcript level.

Introduction

Sialomucin complex (SMC) is a membrane glycoprotein complex isolated from highly metastatic 13762 rat mammary adenocarcinoma cells (Sherblom and Carraway, 1980) and composed of an O-glycosylated mucin subunit ASGP-1 (ascites sialoglycoprotein-1) (Sherblom *et al.*, 1980) and an N-glycosylated integral membrane glycoprotein subunit ASGP-2 (Hull *et al.*, 1990). The latter has two epidermal growth factor-like domains and can act as an intramembrane ligand and modulator of the receptor tyrosine kinase ErbB2 (Carraway *et al.*, 1999). SMC is transcribed from a single gene as a 9-kilobase transcript (Sheng *et al.*, 1992) and translated into a single ≈ 300 kDa polypeptide, which is proteolytically cleaved into the two associated subunits early in its transit to the cell surface (Sheng *et al.*, 1990). Mature glycosylated ASGP-1 has a molecular mass of >500 kDa, with a polypeptide molecular mass of ≈ 220 kDa, and comprises three domains (Wu *et al.*, 1994): an N-terminal unique sequence, a large tandem repeat region rich in serine and threonine residues similar to that of other mucins, and a C-terminal unique sequence. ASGP-2 is a 120-140 kDa protein containing seven domains: two hydrophilic N-glycosylated regions, two epidermal growth factor (EGF)-like domains, a cysteine-rich domain, a transmembrane domain, and a small cytoplasmic domain (Sheng *et al.*, 1992). Recently, it was demonstrated that the N-terminal amino acid sequence of ASGP-1 and the complete sequence of ASGP-2 from rat SMC are 60-70% identical to the sequence of human MUC4 (Moniaux *et al.*, 1999), which contains all of the domains of ASGP-2. Rat SMC differs from human MUC4 by the absence of a 16-amino acid tandem repeat, which was the original identifying characteristic of MUC4 (Porchet *et al.*, 1991). In this presentation, we will use the hybrid term SMC/Muc4.

In tumor cells SMC/Muc4 may have multiple functions. 1) ASGP-1 can provide anti-recognition and anti-adhesive properties to tumor cells (Komatsu *et al.*, 1997), which protect them from killing by natural killer cells (Komatsu *et al.*, 1999) and promote their metastasis (Komatsu *et al.*, 2000). 2) The two EGF-like domains of ASGP-2 have all of the consensus residues present in active members of the EGF growth factor family (Sheng *et al.*, 1992); ASGP-2 has been shown to bind and modulate the receptor tyrosine kinase ErbB2 (Carraway *et al.*, 1999). The interaction between ASGP-2 and ErbB2 may play a role in the constitutive phosphorylation of ErbB2 in the 13762 ascites cells (Juang *et al.*, 1996) and in tumor progression of mammary tumors (Komatsu *et al.*, 1999).

SMC/Muc4 is expressed in a number of normal secretory epithelial tissues in the adult rat, including the mammary gland (Rossi *et al.*, 1996), small and large intestine (Rossi *et al.*, 1996; McNeer *et al.*, 1997), trachea (McNeer *et al.*, 1998a), and uterus (McNeer *et al.*, 1998b), and is probably involved in multiple functions in these organs (McNeer *et al.*, 1997). In mammary gland, SMC/Muc4 is developmentally regulated (Price-Schiavi *et al.*, 1998). It is abundant in milk and lactating mammary gland, but its level is low in the virgin gland. RNA analyses indicate that this differential results from a posttranscriptional effect, since mammary gland RNA levels are similar in virgin and pregnant animals (Price-Schiavi *et al.*, 1998). Upon removal from virgin rat, epithelial cells of mammary glands in culture produce SMC/Muc4, which suggests mammary epithelial cells of virgin rats have the potential to make SMC/Muc4 but their *in vivo* microenvironment prevents them from doing so. In support of this proposal, the reconstituted basement membrane Matrigel and transforming growth factor- β (TGF β) are able to negatively regulate expression of SMC/Muc4 *in vitro* by posttranscriptional mechanisms (Price-

Schiavi *et al.*, 1998; Price-Schiavi *et al.*, submitted).

Although SMC/Muc4 expression is largely post-transcriptionally regulated in the mammary gland, there must be a mechanism *in vivo* for inducing and maintaining expression of SMC/Muc4. In previous studies we have shown that serum stimulates the expression of SMC/Muc4 in isolated mammary epithelial cells (Price-Schiavi *et al.*, 1998). To investigate the factors and pathways involved in this stimulation, we have examined the effect of several serum factors as well as inhibitors and activators of signaling pathways. The results demonstrated the ability of insulin and IGF to upregulate SMC/Muc4 expression at the transcript level. Furthermore, the extracellular-regulated protein kinase (ERK) sub-family of MAP kinases were shown to be involved in the effects of these factors. Together with previous studies of TGF β posttranscriptional downregulation of SMC/Muc4, these results indicate that SMC/Muc4 expression is tightly regulated in mammary gland.

Results

Time course of SMC/Muc4 induction in cultured rat mammary epithelial cells (MEC)

Epithelial cells from virgin rat mammary gland express low levels of SMC/Muc4 protein. We had previously shown that MEC cultured in the presence of 10% fetal calf serum (FBS) have substantially higher levels of SMC/Muc4 than those cultured under serum-free conditions (Price-Schiavi *et al.*, 1998). To determine the timing of SMC/Muc4 induction by serum, MEC were isolated from virgin rats and cultured on plastic in Ham's F-12 medium supplemented with 10% FBS. Cells were harvested at times ranging from 0 to 48 hours and were analyzed by immunoblotting with mAb 4F12 (Rossi *et al.*, 1996). In all tissues studied to date, we have found ASGP-1 and ASGP-2 together in a complex and thus, we assay for ASGP-2 as a marker for SMC/Muc4. Freshly isolated MEC had low levels of SMC/Muc4, as expected. SMC/Muc4 levels began increasing between 3 and 6 hours and increased steadily up to at least 48 hours (Figure 1). These data suggest that one or more factors in serum are involved in maximal induction of SMC/Muc4 protein levels.

Effect of exogenous factors on SMC/Muc4 levels in normal MEC

To determine which factors in serum may be involved in the upregulation of SMC/Muc4, primary mammary epithelial cell cultures were treated with a number of factors demonstrated to have an effect on mammary gland development or differentiation. MEC were isolated and cultured as described in the presence or absence of each factor for 48 hours, followed by immunoblot analysis with anti-ASGP-2 mAb 4F12. Basal levels of SMC/Muc4 protein were determined from MEC cultured in media alone. Fetal bovine serum (FBS) had the largest effect, increasing SMC/Muc4 protein levels by 27-fold, as expected from the large number of components in serum that can potentially act additively to upregulate SMC/Muc4. Individually, insulin and IGF-1 were able to enhance SMC/Muc4 levels by approximately 6-7 fold and may play a significant role in the effect of FBS (Figure 2A). However, growth factors EGF, TGF α , and NDF had no effect on SMC/Muc4 levels in this system, even though these have been shown to play a role in mammary gland development and differentiation (Coleman and Daniel, 1990; Spitzer *et al.*, 1995; Marte *et al.*, 1995). Although estrogen and progesterone have been shown to

affect SMC/Muc4 levels in the uterus (McNeer *et al.*, 1998b), neither hormone affected SMC/Muc4 in cultured MEC. Hydrocortisone was the only hormone observed to have a significant positive effect, increasing SMC/Muc4 levels by approximately 12-fold. Enhanced levels of SMC/Muc4 in cells treated with IGF-1 or insulin suggests that these factors are specifically involved in the regulation of SMC/Muc4 expression in normal rat mammary gland. The lack of SMC/Muc4 response to other factors tested suggests that either they are not involved in SMC/Muc4 regulation or that they are part of larger, more complex pathways that may require synergistic actions with other components or cellular interactions with the surrounding mammary stroma.

Effects of signal transduction pathway inhibitors on SMC/Muc4 expression in normal MEC

We have shown that SMC/Muc4 is regulated largely by post-transcriptional mechanisms during the reproductive cycle in normal rat mammary gland (Price-Schiavi *et al.*, 1998; Price-Schiavi *et al.*, submitted). However, there must be a regulatory mechanism to initiate and maintain production of SMC/Muc4 message prior to pregnancy and in MEC. To address this issue, inhibitors and activators of three types of signaling pathways, tyrosine kinase, protein kinase C, and cyclic AMP, were used to determine if these pathways modulated SMC/Muc4 expression in MEC *in vitro*. MEC were isolated and cultured in Ham's F-12 supplemented with insulin, transferrin, sodium selenite, or 10% FBS and each of the inhibitors to determine what effect, if any, each would have on FBS or insulin stimulation of SMC/Muc4 protein levels in the MEC. After 24 hours cells were harvested and 5 µg total cell protein were subjected to immunoblot analysis with mAb 4F12. SMC/Muc4 protein levels for each treatment were compared to SMC/Muc4 levels from untreated cells cultured under otherwise similar conditions. Thus, treated cells cultured in insulin, transferrin and sodium selenite were compared to untreated cells cultured in the same conditions. SMC/Muc4 levels were reduced by greater than 75% in cells treated with staurosporine, a promiscuous protein kinase inhibitor with effects on protein kinase C, protein kinase A, and tyrosine kinases (Tamaoki *et al.*, 1986). Tyrphostin A23, a more specific inhibitor of tyrosine kinases that acts on their substrate sites (Levitski *et al.*, 1991) also inhibited SMC/Muc4 expression by greater than 75%. However, genistein, which inhibits tyrosine kinases by binding to the ATP-binding site (Akiyama *et al.*, 1987), had no effect on SMC/Muc4 levels under these conditions. Northern blot analysis of total RNA extracted from cells treated with staurosporine revealed that this inhibitor also reduces SMC/Muc4 transcript levels (data not shown). The protein kinase C activator phorbol 12-myristate 13-acetate had no effect on SMC/Muc4 levels. None of the agents that elevate cellular cyclic AMP, dibutyryl cyclic AMP, forskolin, and isobutylmethylxanthine, had an effect on SMC/Muc4 levels. Finally, neither sodium vanadate nor okadaic acid, which inhibit protein phosphatases, had any effect on SMC/Muc4 levels in these assays. Taken together, these data suggest that the signaling pathway(s) regulating basal SMC/Muc4 expression requires tyrosine phosphorylation and does not seem to involve some protein phosphatases. We can not at this time rule out the possibility that the protein kinase C/A pathways may be involved in SMC/Muc4 expression in these cells.

Role of ERK signaling pathway in expression of SMC/Muc4

Up-regulation of SMC/Muc4 initiated by FBS, IGF-1 and insulin correlated with activation of the ERK sub-family of MAP kinases (Figure 2B). MEC were isolated and treated with either FBS, IGF-1, or insulin. Cells were then analyzed for SMC/Muc4 expression by immunoblot analysis with mAb 4F12 and for ERK activity by an ERK kinase assay or by immunoblot analyses with anti-phospho-ERK and anti-ERK. Cells treated with FBS had the highest levels of SMC, while cells cultured under serum-free conditions had the lowest (Figure 2A). Cells treated with IGF-1 or insulin had intermediate levels of SMC. All of the cultured MEC express ERK1 and ERK2; however, ERK1 and ERK2 were activated (phosphorylated) only in cells treated with FBS, IGF-1, or insulin. ERK1 and ERK2 were not activated (phosphorylated) in control cells cultured under serum-free conditions. We were unable to detect any activity of p38 in MEC cultured under these conditions (data not shown). These data suggest that IGF-1 and/or insulin signaling through the ERK pathway could be involved in the induction of SMC/Muc4 by FBS.

To determine whether the ERK pathway is involved in up-regulation of SMC/Muc4 by growth factors, virgin MEC were incubated with the MEK-1 inhibitor U0126 at different concentrations for 30 minutes, followed by culture in serum-containing medium for 24 hours. U0126 inhibited SMC/Muc4 expression in a dose-dependent manner, in parallel with its inhibition of ERK activation (Figure 3A). To determine if other MAP kinase family members are also involved in regulation of SMC/Muc4 expression, the inhibitory effect of the MEK-1 inhibitor U0126 on SMC/Muc4 expression was compared to that of p38 MAP kinase inhibitor SB203580. Cells were treated under the conditions described above and analyzed by immunoblotting with mAb 4F12, anti-ERK, or anti-phospho-ERK antibodies. In contrast to the inhibitory effect of U0126 on both SMC/Muc4 expression and activation of ERK1 and ERK2, SB203580 had no effect on SMC/Muc4 expression or ERK1/ERK2 activation (Figure 3B). A control reagent, SB202474, which does not inhibit MAP kinases, also had no effect on SMC/Muc4 or ERK1/ERK2 activation. These data indicate that activation of ERK, but not p38 MAP kinase, is required for up-regulation of SMC/Muc4 by FBS (growth factors).

To determine if activation of the ERK pathway is also involved in induction of SMC/Muc4 by insulin or IGF-1, MEC were treated with U0126 and analyzed as described above. In cells treated with either insulin or IGF-1 alone, ERK activity was stimulated and SMC/Muc4 levels were higher than in untreated cells (Figure 4). ERK activity and induction of SMC/Muc4 by insulin or IGF-1 were blocked by treatment with U0126 with no effect on the overall levels of ERK1 or ERK2 protein. These data suggest that insulin and IGF-1 are partly responsible for the induction of SMC/Muc4 by serum, and that activation of the ERK signaling pathway is involved in regulation of SMC/Muc4 by these growth factors.

Requirement for sustained ERK Activity for induction of SMC/Muc4

EGF family members and IGF-1 have been shown to be involved in mammary gland development (Taketani and Oka, 1983a,b; Kleinberg, 1998). EGF, PDGF, and IGF-1 are all able to activate ERK (Boulton *et al.*, 1991; Cahill and Perlman, 1991; Thomas, 1992). However, IGF-1 and insulin, but not EGF or PDGF, are able to induce SMC/Muc4 expression in cultured MEC. Signaling through the IGF-1 receptor has been reported to cause sustained activation of ERK, while signaling through the EGF receptor causes only transient activation (Swantek and

Baserga, 1999). To determine whether transient or sustained ERK activation is involved in upregulation of SMC, cultured MEC were treated with either EGF, PDGF, IGF-1, insulin, or serum. Cells were harvested at times ranging from 10 minutes to 24 hours after the beginning of growth factor treatment, and the duration of ERK activation by each of the growth factors was measured by immunoblotting with anti-phospho-ERK. ERK was activated at 10 minutes by all of the growth factors as well as FBS (Figure 5A), but ERK activity was sustained for 24 hours only in cells treated with IGF-1, insulin or FBS (Figure 5B). The sustained activation of ERK by IGF-1, insulin or FBS correlates with the induction of SMC/Muc4. To determine what length of sustained ERK activity is required for up-regulation of SMC/Muc4, cultured MEC were incubated with F-12 medium containing 10% FBS for times ranging up to 9 hours. U0126 was added at 3, 6 and 8 hours to block ERK activation, and cells were collected and analyzed by immunoblotting. Only cells treated with U0126 after at least a 6-hour incubation with FBS were able to express higher levels of SMC/Muc4 (Figure 5C). Thus, ERK activity must be sustained for at least 6 hours to achieve maximal induction on SMC/Muc4 by serum.

To demonstrate further that sustained ERK activity will up-regulate SMC/Muc4, hemagglutinin(HA)-MEK-1 (S218/222D), a constitutively activated form of MEK-1 carrying an HA epitope tag, was transiently transfected into 13762 MAT-BIII cells, a cultured variant of the 13762 ascites tumor which also express SMC/Muc4 (Figure 5D). Transfected cells were harvested and analyzed by immunoblotting with mAb 4F12, anti-HA, anti-ERK, and anti-phospho-ERK antibodies. When activated MEK-1 was overexpressed, ERK activity and SMC/Muc4 expression were somewhat higher than in vector-transfected control cells. The MAT-BIII cells are particularly difficult to transfect, and transfection efficiency tend to be low in these cells. Thus, the increase in SMC/Muc4 expression in cells transfected with the activated MEK-1 would be more evident with higher transfection efficiency. Nonetheless, there was an increase in SMC levels in these cells when activated MEK-1 was overexpressed. Taken together these data indicate that sustained ERK activity is involved in upregulation of SMC/Muc4 by serum factors.

ERK signaling pathway and transcript levels of SMC/Muc4

We have shown previously that SMC/Muc4 is post-transcriptionally regulated by both Matrigel (ECM) and TGF β in normal rat MEC (17). However, there must also be a pathway to regulate transcription of SMC/Muc4 in normal MEC. As described above, tyrosine phosphorylation is required for maintenance of both SMC/Muc4 protein and transcript levels, and ERK activity is required for maximal induction of SMC/Muc4 by serum in cultured MEC. Steady state levels of SMC/Muc4 mRNA from MEC treated with either FBS and/or U0126 were analyzed by Northern blots to determine if ERK activation is involved in transcriptional or post-transcriptional regulation of SMC/Muc4. The SMC/Muc4 transcript was induced about 2-fold over untreated control cells after virgin rat MEC were incubated with serum for 24 hours (Figure 6A and B). However, SMC/Muc4 mRNA was not increased when cells were treated with both serum and U0126. This result suggests that regulation of Muc4 expression by the ERK signaling pathway occurs partly by some modulation of SMC/Muc4 transcript level.

Discussion

Developmental regulation of SMC/Muc4 in normal rat mammary gland is complex, involving several different regulatory mechanisms. Among these must be a mechanism to initiate and maintain transcription and basal expression of SMC/Muc4 in the normal animal. We have previously demonstrated posttranscriptional and posttranslational mechanisms for negatively regulating SMC/Muc4 protein levels (Price-Schiavi *et al.*, 1998; Price-Schiavi *et al.*, 2000). Here we report positive regulation of SMC/Muc4 by insulin and IGF-1. Regulation of SMC/Muc4 by both of these growth factors requires activation of the ERK signaling pathway and affects SMC/Muc4 expression at the transcript level.

Both IGF-1 and insulin have been shown to have important roles in mammary gland development and differentiation. IGF-1 plays a role in pubertal mammary gland development, most likely by mediating the effects of growth hormone (Kleinberg, 1998). IGF-1 applied directly to the mammary glands of hypophysectomized, oophorectomized, sexually immature rats simulates substantial ductal growth and terminal end bud formation (Ruan *et al.*, 1992; Ruan *et al.*, 1995). Furthermore, IGF-1 mice have significantly reduced mammary development, which can be partially reversed by treatment with IGF-1 (Ruan *et al.*, 1999). There is also a proposed role for IGF-1 in later stages of mammary development. Overexpression of IGF-1 in the mammary glands of transgenic mice during lactation leads to ductal hypertrophy and inhibits involution (Hadsell *et al.*, 1996; Neuenschwander *et al.*, 1996). Insulin is essential for milk protein gene expression and cell survival in the mammary gland. *In vitro* insulin is required for maintaining mammary tissue in culture and stimulates phosphoprotein synthesis (Voytovich *et al.*, 1969). Insulin has also been shown to stimulate expression of α -lactalbumin and β -casein (Nicholas *et al.*, 1983; Prosser *et al.*, 1987). IGF-1 can similarly stimulate milk protein synthesis but is only about one-tenth as potent as insulin in this regard. Both growth factors have been shown to be equally involved in stimulation of DNA synthesis and inhibition of apoptosis in HC11 mammary epithelial cells (Merlo *et al.*, 1996; Wartmann *et al.*, 1996).

SMC/Muc4 is expressed at low levels in virgin mammary gland, increases sharply during pregnancy reaching maximum levels during lactation, and decreases rapidly during involution (Price-Schiavi *et al.*, 1998). Although the exact timing is as yet unknown, initiation of SMC/Muc4 expression must occur very early during mammary development because it is detectable in the rudimentary ductal tree (ducts and terminal end buds) of 3 week old prepubertal female rats (Li and Carraway, unpublished). In cultured MEC, FBS substantially enhances both SMC/Muc4 protein and mRNA levels, suggesting that some factor or factors in serum may be involved in regulating SMC/Muc4 expression. Of the factors tested under our culture conditions, insulin and IGF-1 could substantially reproduce the increase in SMC/Muc4 levels produced by serum. The presence of IGF-1 and insulin in the mammary gland and their effects on mammary gland development and differentiation suggest that both of these factors may be involved in initiation and maintenance of SMC/Muc4 expression in normal rat mammary gland. The fact that all three factors (serum, insulin, and IGF-1) stimulate the ERK signalling pathway in a similar manner suggests that insulin and IGF-1 are at least partially responsible for the serum effect.

Inhibition of the ERK pathway by the MEK-1 inhibitor U0126 led to decreased SMC/Muc4 protein and transcript levels. These observations suggest that the ERK signaling pathway (through IGF-1 and/or insulin) is involved in maintenance of SMC/Muc4 expression at

the transcript level. The downregulation of SMC/Muc4 transcript with U0126 treatment is not complete after 24 hours. This suggests that the SMC/Muc4 message has a slow turnover and/or that regulation of SMC/Muc4 via an ERK dependent pathway also involves post-transcriptional mechanisms. A primary function of the ERK signaling pathway involves the regulation of transcription factors. Downstream targets of ERK1/2 include ribosomal S6 kinase (RSK) (Waskiewicz and Cooper, 1995), ternary complex factors (TCFs) such as Elk-1, and signal transducer and activator of transcription (STAT) family members, such as STAT5a (McCawley *et al.*, 1999; Marais, *et al.*, 1993). Furthermore, RSK has been shown to regulate transcription factors such as c-Fos, estrogen receptor, NFkB/IkB α , c-AMP-response element-binding protein (CREB) and CREB-binding protein (Frodin and Gammeltoft, 1999). We have recently cloned and sequenced a putative rat SMC/Muc4 promoter (Price-Schiavi *et al.*, submitted). This sequence contains several transcription factor-binding sites, including AP-1, NFkB, CREB and STAT5. Studies are currently underway to determine which, if any, of these sites are involved in regulation of SMC/Muc4 expression downstream of ERK1/2 activity.

Cellular behavior can be determined by the duration of ERK activity. For example, in keratinocyte cell lines, ligands that transiently activate p42/p44 ERK/MAP kinases are mitogenic but do not induce MMP-9 expression or colony dispersion (McCawley *et al.*, 1999). On the other hand, ligands that induce sustained activation of these kinases stimulate MMP-9 expression and colony dispersion (McCawley *et al.*, 1999). For enhancement of SMC/Muc4 levels by serum, sustained activity of ERK is necessary. Although all of the growth factors we tested (IGF-1, insulin, EGF, and PDGF) are capable of activating the ERK pathway, IGF-1 and insulin are the only ones that could elicit sustained activation of ERK1/2 under these conditions (Boulton *et al.*, 1991; Cahill and Perlman, 1991; Thomas, 1992). In our culture system, only IGF-1 or insulin, which induce sustained activation of ERK1/2, could partially mimic the high levels of SMC/Muc4 induced by serum. In contrast, EGF and PDGF, which transiently activate ERK1/2, could not. Thus, we propose that stimulation of IGF-1 or insulin receptor(s) by IGF-1 or insulin induces sustained activation of ERK, which in turn promotes upregulation of expression of the SMC/Muc4 transcript.

Our combined studies indicate that regulation of SMC/Muc4 in normal mammary gland is very complex. The ERK pathway (serum, insulin, and IGF-1) is part of a complicated equation for maintenance of SMC/Muc4 expression, because absence of these factors or inhibition of the ERK pathway does not completely abolish SMC/Muc4 expression. Treatment of MEC with inhibitors such as staurosporine or tyrphostin A23 drastically reduce SMC/Muc4 expression at both the protein and transcript level, suggesting that other pathways involving tyrosine kinases may be involved in its regulation. These data allow us to propose the following model. Growth factor signaling through the Ras, Mek-1, ERK pathway acts to upregulate and maintain expression of SMC/Muc4 transcript and protein in normal MEC. SMC/Muc4 protein levels are modulated by post-transcriptional repression by TGF β and the extracellular matrix. Loss of the repression by TGF β and extracellular matrix are necessary for full expression of SMC/Muc4 as a milk protein. However, loss of these repressive mechanisms in conjunction with neoplastic transformation and activation of the ERK pathway could facilitate tumor progression via the anti-recognition (Komatsu *et al.*, 1999), anti-adhesion (Komatsu *et al.*, 1997) and ErbB2-modulating (Carraway *et al.*, 1999) mechanisms of SMC/Muc4. Thus, in a sense, the

tumor cell is able to "hijack" the mechanism for expression of the milk protein for its own purposes. One of those purposes is likely to use the protective functions of the mucin (McNeer *et al.*, 1997) to protect the tumor cell from being killed by the immune system (Komatsu *et al.*, 1999).

Materials and methods

Materials

U0126 was purchased from Promega (Madison, WI). SB203580, SB202474, actinomycin D, tunicamycin and cycloheximide were from Calbiochem (San Diego, CA). Prolactin and hydrocortisone were purchased from Sigma (St. Louis, MO). Polyclonal (Sheng *et al.*, 1990) and monoclonal (Rossi *et al.*, 1996) antibodies against ASGP-2 were made as previously described. Anti-ERK was purchased from Zymed (South San Francisco, CA). Anti-phospho-ERK was from Promega and Calbiochem. Anti-HA was a gift from Dr. Catherine Welsh, University of Miami. TGF β , TGF α , EGF, IGF-1 and insulin were all from R & D Systems (Minneapolis, MN). Plasmid pCMV-HA/Mek-1 (S218/222D) was a gift from Dr. Michael Weber, University of Virginia.

Cell culture and transfection

Mammary epithelial cells (MEC) from virgin female Fischer 344 rats were prepared as previously described (Price-Schiavi *et al.*, 1998) and cultured in F-12 medium (or phenol red-free Ham's F-12 when cells were treated with estrogen or progesterone) with FBS, insulin-like growth factor-I, or insulin for indicated times. For experiments with kinase inhibitors and TGF β , freshly isolated MEC were pretreated with the inhibitors in serum-free F-12 medium for 0.5 hour and then incubated with F-12 medium containing 10% FBS, IGF-I, or insulin for 24 hours. (Still not clear) The MAT-BIII cultured subline of the 13762 rat mammary adenocarcinoma was maintained in McCoy's medium with 10% FBS. For transfection, 5×10^5 MAT-BIII cells were seeded in 6-well plates on day 1. On day 2, cells were transfected with 2 μ g/well of HA-MEK-1 (S218/222D) or control vector with lipofectamine (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's procedure. Cells were collected and extracted for immunoblotting on day 3.

Immunoblot analyses

MEC and MAT-BIII cells were collected by scraping after the indicated treatment. Cells were extracted in NP-40 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 5 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1/100 diluted protease inhibitor cocktail) as previously described (Zhu and Assoian, 1995; Zhu *et al.*, 1996). Cell extracts (10-15 μ g protein) were analyzed in 7.5% polyacrylamide gels with the mini-Protean II system (Bio-Rad, Hercules, CA). Resolved proteins were transferred to nitrocellulose membranes and blocked with 3-5% non-fat milk (Bio-Rad) in TBS with 0.1% Tween-20 and 0.1% sodium azide. The membranes were then probed with first and second antibodies. Proteins were detected by the RenaissanceTM Enhanced Chemiluminescence kit (NEN Life Science Products Inc, Boston, MA). For comparisons of SMC/Muc4 protein levels

with various treatments, the ASGP-2 (SMC/Muc4) band from each sample was quantified by densitometry and the ratio was taken of ASGP-2 (SMC/Muc4) from the treated versus the untreated samples.

Northern blots

MEC were collected by scraping and extracted in Trizol Reagent (Life Technologies, Inc) according to the manufacturer's procedure. Total RNA (10-15 µg) was electrophoresed on 1% formaldehyde/agarose gels. Fractionated RNA was transferred to Hybond-N nylon membranes (Amersham), followed by cross-linking using a Stratalinker (Stratagene, La Jolla, CA). The membranes were prehybridized for 3-5 hours at 42°C in prehybridization solution (50% formamide, 1M NaCl, 2x Denhardt's solution, 1% SDS, 0.1 g/ml dextran sulfate, and 0.5 mg/ml salmon sperm DNA). The probes, A2G2-9, a 1.7-kb probe which spans the 5' unique region and four tandem repeats of SMC/Muc4 cDNA, and cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were random prime-labeled with [³²P]dCTP using a Random Primed Labeling Kit (Boehringer-Mannheim, Indianapolis, IN). The membranes were hybridized overnight at 42°C in prehybridization solution containing labeled probes. Following hybridization, membranes were washed 3 times at room temperature in 2 x SSC with 0.1% SDS for 30 minutes each, and 3 times at 50°C in 0.1 x SSC with 0.1% SDS for 30 min each. Radioautography was performed on Kodak LS x-ray films (VWR, Atlanta, GA). For comparisons of SMC/Muc4 steady state RNA levels, the SMC/Muc4 band and the GAPDH band from the Northern blot were quantified by densitometry. The ratio of SMC/Muc4 to GAPDH was calculated and the results were plotted.

Immunoprecipitation and in vitro ERK kinase assays

The kinase assay was performed as previously described (Chen *et al.*, 1994) with minor modifications. Briefly, 50 µg of cell extracts were immunoprecipitated by anti-ERK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at 4°C, and the resulting immune complex was incubated with protein A-agarose (Sigma) for an additional 2 hours. Immobilized immune complexes were washed twice with 0.25 M Tris, pH 8.0, once with 0.1 M NaCl and 50 mM HEPES, pH 8.0, and once with 25 mM HEPES, pH 8.0, 10 mM MgCl₂. The immunoprecipitated ERK was incubated with kinase assay mixture (25 mM HEPES, pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol, 0.5 mg/ml myelin basic protein, 50 mM ATP, and 10 mCi of [γ -³²P] ATP) in a final volume of 30 µl at room temperature for 30 minutes. The reaction was stopped by adding 30 µl of 2x SDS sample buffer and boiling for 5 minutes. The samples were centrifuged, and supernatants were collected for electrophoresis on a 15% polyacrylamide gel. After staining with Coomassie Brilliant Blue, the gel was dried and exposed to Kodak LS x-ray film at -70°C.

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FIGURE LEGENDS

Figure 1 Time course of SMC/Muc4 induction in normal cultured MEC. MEC were isolated and cultured in Ham's F-12 medium supplemented with 10 % FBS (Price-Schiavi *et al.*, 1998). Cells were harvested at times ranging from 0 to 48 hours as indicated at the bottom of the figure, and 15 μ g total protein for each time point analyzed by immunoblotting with mAb 4F12.

Figure 2 Effect of IGF-1 and insulin on SMC/Muc4 expression in normal cultured MEC. Cultured MEC were treated with 50 ng/ml IGF-1, 5 μ g/ml insulin, or 10% FBS for 24 hr. Cells were extracted and analyzed by immunoblotting with anti-ASGP-2 mAb 4F12, anti-ERK, or anti-phospho-ERK antibodies, or by the ERK kinase assay, as indicated at the right of the figure.

Figure 3 Effect of U0126 MEK-1 kinase inhibitor on SMC/Muc4 levels in cultured MEC. A) MEC were preincubated with 0-80 μ M of U0126, a MEK-1 inhibitor, for 0.5 hour and then cultured in 10% FBS-containing medium for 24 hours. B) MEC were preincubated with dimethylsulfoxide carrier solvent control, 40 μ M U0126, 40 μ M SB203580, or 40 μ M SB 202474 for 30 minutes, followed by culture in 10% FBS-containing medium for 24 hours. Cells were extracted and subjected to immunoblot analysis with mAb 4F12, anti-ERK, or anti-phospho-ERK antibodies as indicated at the right of each panel.

Figure 4 Effect of U0126 on SMC/Muc4 induction by IGF-1 and insulin in normal cultured MEC. MEC were incubated in the presence or absence of 40 μ M U0126, followed by culture in Ham's F-12 medium supplemented with either 50 ng/ml IGF-1 or 5 μ g/ml insulin for 24 hours as indicated at the top of the figure. Cells were collected, extracted, and analyzed by immunoblotting with anti-ASGP-2, anti-phospho-ERK and anti-ERK as indicated at the right of the figure.

Figure 5 Sustained activation of ERK is necessary for up-regulation of SMC/Muc4. A) MEC were untreated (control) or treated with EGF (20 ng/ml), PDGF-BB (10 ng/ml), IGF-1 (50 ng/ml), insulin (5 μ g/ml), or FBS (10%) for 10 minutes. B) MEC were treated with the same reagents as in panel A for 24 hours. C) MEC were incubated in F-12 medium containing 10% FBS without or with U0126 (80 μ M) added at times 0, 3, 6, or 8 hours. The total incubation time was 9 hours. Cells in A-C were extracted and analyzed by western blotting with anti-phospho-ERK, anti-ERK and anti-ASGP-2. D) Rat mammary epithelial tumor cell line MAT-BIII cells were transiently transfected with HA-tagged MEK-1 (S218/222D) (+) or vector only (-) in F-12 medium containing 0.5% FBS. Twenty-four hours after transfection, cells were collected and extracted. The cell extracts were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-ASGP-2, anti-HA, anti-phospho-ERK and anti-ERK.

Figure 6 SMC/Muc4 transcript level regulation through ERK signaling pathway. A) MEC were isolated from virgin rat mammary glands and treated with or without 10% FBS and 80 μ M U0126 for 0.5 hour and then incubated with 10% FBS for 24 hours. Cells were extracted and the total RNA was isolated. 10 μ g/lane total RNA was resolved and analyzed by Northern blot with

probe A2G2-9. The same blots were also probed with a c-DNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. B) Plot of SMC/Muc4 transcript levels in cultured MEC or U0126 treated MEC. The bands for SMC/Muc4 and GAPDH were quantified by densitometry. The values for the SMC/Muc4 bands were divided by the values for the corresponding GAPDH bands and the results were plotted.

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 (TGF β). The repression of SMC expression by TGF β is rapid, is independent of TGF β -induced cell cycle arrest, and does not require new protein synthesis. Unlike Matrigel, TGF β does not reduce SMC protein synthesis, as SMC precursor accumulation is equivalent in TGF β -treated and untreated cells. Instead, SMC precursor in TGF β -treated cells is more persistent and does not become processed as rapidly into mature ASGP-1 and ASGP-2, indicating that TGF β disrupts processing of SMC precursor. These results indicate that SMC, a product of normal mammary gland and milk, is regulated by TGF β by a novel post-translational mechanism. Thus, SMC is regulated by multiple post-transcriptional mechanisms, which serve to repress potential deleterious effects of overexpression.

TGF β ¹ 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, TGF β implants introduced into the mammary glands of subadult virgin mice can inhibit ductal development (1). In addition, overexpression of TGF β 1 in the mammary glands of transgenic mice inhibited lobuloalveolar development and milk protein production (2). TGF β can induce expression of extracellular matrix proteins by human mammary epithelial cells in culture (3). Further, TGF β 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, TGF β appears to play a role in regulating accumulation of milk proteins during pregnancy.

TGF β 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 MUC4 β , 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-

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¹ The abbreviations used are: TGF β , transforming growth factor β ; SMC, sialomucin complex; ASGP, ascites sialoglycoprotein; MEC, mammary epithelial cell; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; mAb, monoclonal antibody; MAP, mitogen-activated protein.

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. TGF β 1 can also regulate SMC levels in normal cultured mammary epithelial cells, but not the ascites tumors, by a post-transcriptional mechanism (6).

In the present study, we have characterized the mechanism of post-transcriptional regulation of SMC by TGF β in cultured primary mammary epithelial cells. TGF β inhibits induction of SMC expression when the cells are put into culture; the repression of SMC expression is rapid and is independent of TGF β -induced cell cycle arrest. The presence of TGF β 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, TGF β has little effect on SMC precursor synthesis. Instead, TGF β alters the processing of SMC precursor into mature SMC (ASGP-1/ASGP-2), a novel TGF β action, which appears not to be a consequence of the effects of TGF β 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 immunoblots (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-smooth muscle actin mouse monoclonal antibody was purchased from Sigma. TGF β 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 materials were obtained from Life Technologies, Inc., unless otherwise noted.

Cell Culture and Analysis—Primary mammary epithelial cell cultures were prepared from virgin Fischer 344 female rats by collagenase digestion of dissected mammary tissue. Briefly, mammary glands excised from virgin or pregnant female Fischer 344 rats were minced, resuspended in digestion medium comprising 1 mg/ml collagenase type II (Worthington Biochemical Corp., Freehold, NJ) and 100 units/ml penicillin, 100 μ g/ml streptomycin in Ham's F-12 medium (Life Technologies, Inc.) and incubated at 37 °C with shaking for 45 min. Fully and partially digested epithelial cell clusters were pelleted and incubated a second time in digestion buffer at 37 °C with shaking for 45 min. Digested epithelial cell clusters were pelleted, resuspended in PBS, and passed through a 520- μ m cell sieve to remove undigested material. Mammary epithelial cell clusters in the resulting filtrate were captured on a 70- μ m nylon membrane. Cell clusters were collected by rinsing the membrane with PBS and were subsequently washed three times in PBS prior to plating. Mammary epithelial cells were maintained in Ham's F-12 medium containing 10% FCS and 100 units/ml penicillin, 100 μ g/ml streptomycin. TGF β was added at a final concentration of 200 pM either at the time of plating or after 24 h of culture. Cells were cultured at 37 °C in 5% CO₂ for 48 h prior to harvest. Cells were collected from culture on plastic dishes by scraping cells off the dish. Except where indicated, harvested cells were pelleted, washed with PBS, and lysed in 100 μ l of 1% SDS in water. Protein concentration of the cell lysates was

determined by Lowry assay, and 5 μ g 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-Protein II system (Bio-Rad). Resolved proteins were transferred to nitrocellulose membranes which were subsequently blocked with 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 Renaissance™ 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 TGF β 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 Cys/Met-free Dulbecco's minimal essential medium supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, and 10 mM Hepes, and incubated in 1 ml of labeling medium (starvation medium + 550 μ Ci/ml [³⁵S]Cys + [³⁵S]Met) (EXPRE³⁵S³⁵S 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 lysed in 200 μ l of 2% SDS in H₂O. For pulse-chase studies, labeled cells were washed twice with prelabeled medium and then incubated in Ham's F-12 supplemented with 10% FCS and 200 pM TGF β , where indicated, for times ranging from 0 to 8 h. After labeling, cells were washed twice with PBS and lysed in 200 μ l 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, 190 mM NaCl, 60 mM Tris-HCl, 6 mM EDTA, pH 7.4). Diluted cell lysates were centrifuged at 20,000 $\times$ 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 7.6) 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 μ l 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 TGF β —We have shown previously that SMC/Muc4 protein is induced rapidly when isolated mammary epithelial cells are cultured as a monolayer on plastic tissue culture dishes. Further, we demonstrated that TGF β 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 TGF β . 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. This antibody recognizes both 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 TGF β 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 TGF β . 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 meas-

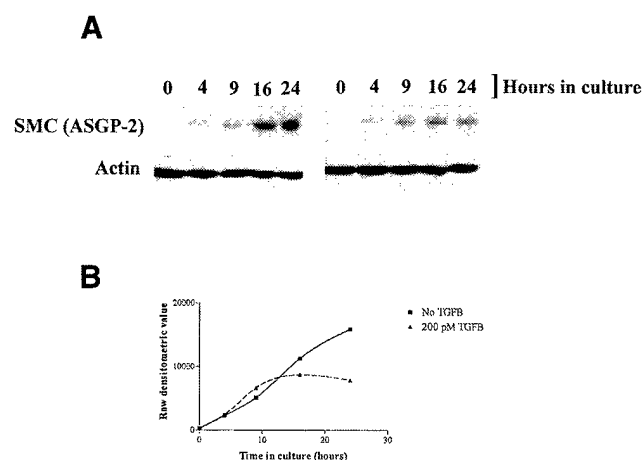


FIG. 1. SMC (ASGP-2) expression in normal rat MEC in the presence or absence of TGFβ. Normal virgin rat MEC were isolated and cultured on plastic in Ham's F-12 medium supplemented with 10% fetal calf serum in the presence or absence of 200 pM TGFβ. Cells were harvested at the times indicated. Cell lysates were prepared for each time, and 5 μg of total protein were subjected to SDS-PAGE and immunoblot 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.

ured as a loading control. In the absence of TGFβ, SMC (ASGP-2) appears at about 4 h after plating and reaches maximal levels only after 24 h (Fig. 1A). In the presence of TGFβ, SMC (ASGP-2) also appears at about 4 h after plating but levels off by about 12 h. The maximal level of SMC (ASGP-2) in MEC cultured in the presence of TGFβ is about 50% of that in cells cultured without TGFβ (Fig. 1B).

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

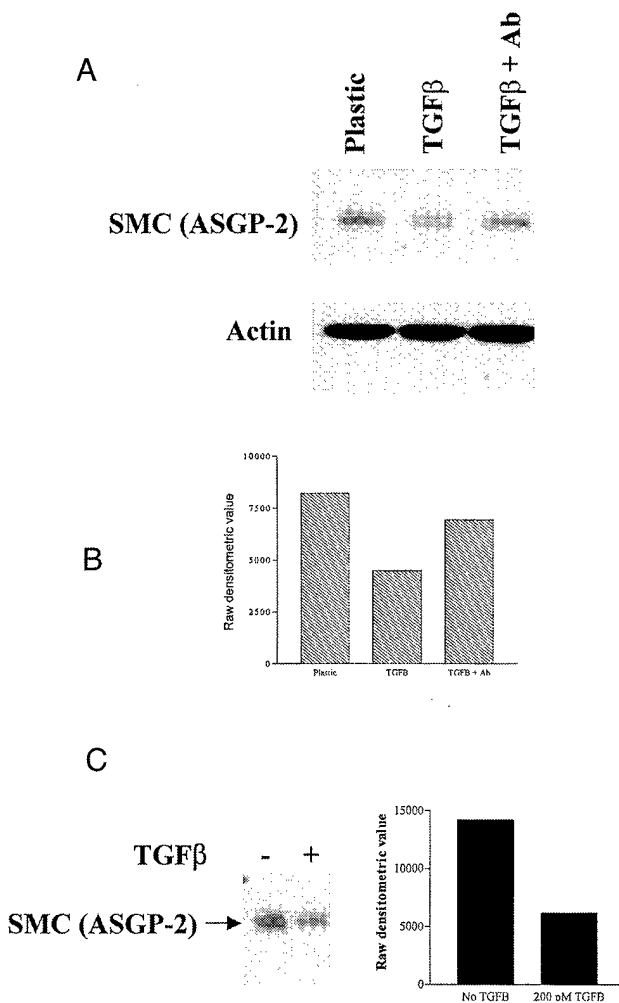


FIG. 2. Specificity of the TGFβ effect on SMC (ASGP-2) expression. Normal virgin rat MEC were isolated and cultured on plastic dishes in Ham's F-12 supplemented with 10% fetal calf serum in the presence or absence of 200 pM TGFβ and an anti-TGFβ neutralizing antibody for 24 h. Neutralizing anti-TGFβ antibody (Ab) was incubated with TGFβ prior to addition to the culture. Cells were cultured, harvested, and analyzed by immunoblot with anti-ASGP-2 mAb 4F12 or anti-actin antibodies as indicated at the left of the figure (A). B, quantitation of SMC (ASGP-2) levels by densitometric analysis of the bands from A. C, after 24 h of culture MEC were harvested and counted. 5.0×10^4 cells were used for immunoblot analysis with anti-ASGP-2 mAb 4F12.

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 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

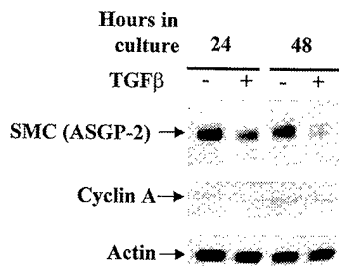


FIG. 3. **Repression of SMC (ASGP-2) by TGFβ is independent of cell cycle arrest.** Normal virgin MEC were isolated and cultured on plastic in Ham's F-12 supplemented with 10% fetal calf serum and 200 pM TGFβ. After 24 or 48 h, cells were harvested and 5 μg of total protein were subjected to immunoblot analysis with mAb 4F12, anti-cyclin A, or anti-actin antibodies as indicated at the left of the figure.

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 radioimmune precipitation 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 pro-

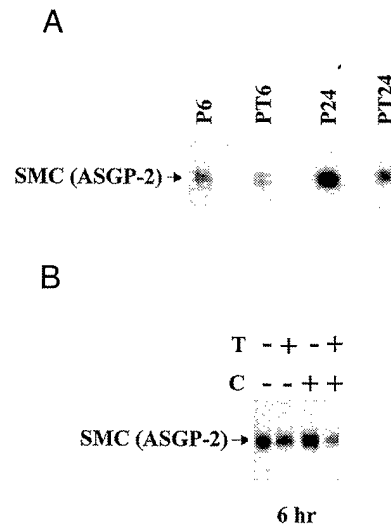


FIG. 4. **Timing and effects of cycloheximide on the repression of SMC (ASGP-2) synthesis by TGFβ.** Normal virgin MEC were isolated and cultured on plastic in medium supplemented with 10% fetal calf serum. After 24 h, the serum-containing medium was replaced and the cells were cultured for an additional 6 or 24 h in the presence or absence of 200 pM TGFβ (A) with or without 10 μg/ml cycloheximide (B). B, cells were harvested and lysates prepared at 6 h as indicated at the top of the figure. 5 μg of total protein was subjected to SDS-PAGE and immunoblot analysis with mAb 4F12. P, plastic; PT, plastic + 200 pM TGFβ; T, 200 pM TGFβ; C, 10 μg/ml cycloheximide.

duced 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),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

² S. A. Price-Schiavi, X. Zhu, R. Aquinin, and K. L. Carraway, unpublished observation.

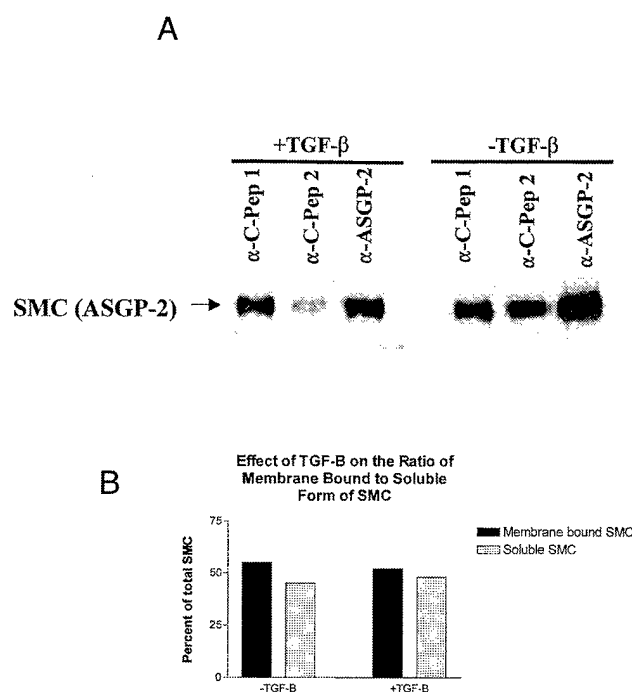


FIG. 5. Effect of TGFβ on the ratio of soluble to membrane-bound forms of SMC (ASGP-2) in normal cultured MEC. Virgin MEC were isolated and cultured in the presence or absence of 200 pM TGFβ. After 48 h cells were harvested and immunoprecipitated twice sequentially with anti-C-Pep to clear the lysate of membrane form. The lysate was then immunoprecipitated with polyclonal anti-ASGP-2 to obtain the remaining SMC (ASGP-2), which was not recognized by anti-C-Pep. Immunoprecipitates were subjected to immunoblot analysis with mAb 4F12. *A*, Western blot of serial immunoprecipitates from MEC cultured in the presence or absence of TGFβ. *B*, percentages of membrane-bound and soluble forms of SMC (ASGP-2) in MEC cultured in the presence or absence of TGFβ. The bands from *A* were quantified by densitometry and the raw values added for total SMC (ASGP-2). Values of both anti-C-Pep bands were added and divided by the total value to obtain the membrane SMC (ASGP-2) percentage. The value of the polyclonal anti-ASGP-2 band was divided by the total value to obtain the soluble form percentage.

TABLE I
Estimated half-life of SMC (ASGP-2) in MEC cultured in the presence or absence of TGFβ

	No TGFβ	200 pM TGFβ
	<i>h</i>	<i>h</i>
Cycloheximide	12	14
Puromycin	10	12
Tunicamycin	12	12

(ASGP-2) precursor and mature ASGP-2. Samples were subjected to SDS-PAGE and fluorography. Total protein synthesis was similar in both treated and untreated samples, indicating that TGFβ did not inhibit total protein synthesis. The amount of accumulating SMC (ASGP-2) precursor detected in both treated and untreated samples was similar for all time points (Fig. 6A). Precursor accumulation was quantified by densitometry (Fig. 6B). These data demonstrate that similar levels of precursor were synthesized in the presence or absence of TGFβ, indicating that TGFβ does not affect the rate of SMC (ASGP-2) precursor biosynthesis (message translation). Thus, the reduction of SMC (ASGP-2) levels by TGFβ involves a different mechanism from that for Matrigel, which inhibits SMC (ASGP-2) precursor biosynthesis (6).

Effect of TGFβ on Processing of SMC (ASGP-2) Precursor—Since TGFβ does not affect SMC translation or the turnover of

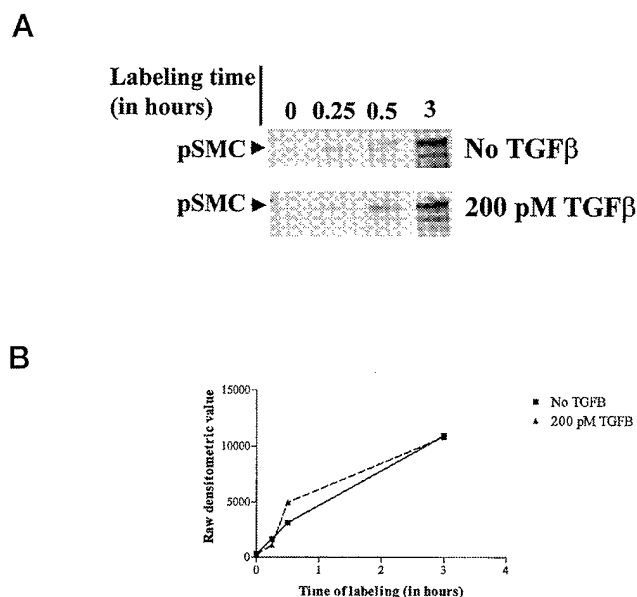


FIG. 6. Effect of TGFβ on SMC (ASGP-2) precursor biosynthesis in normal cultured MEC. *A*, virgin MEC were isolated and cultured in the absence of TGFβ. After 24 h TGFβ (200 pM final concentration) was added to half the samples. After an additional 24 h, cells were metabolically labeled with [³⁵S]Cys + [³⁵S]Met for various times as indicated at the top of the figure. Cells were harvested and immunoprecipitated with anti-ASGP-2 polyclonal antibody (equivalent total counts per time point). Immunoprecipitates were subjected to SDS-PAGE and fluorography. *B*, plot of SMC (ASGP-2) precursor accumulation. The precursor bands from *A* were quantified by densitometry, and the results were plotted.

the mature protein, another possibility is that TGFβ 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 TGFβ 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 prelabeled medium twice and incubated in chase medium for times ranging from 1 to 8 h. TGFβ 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 TGFβ, suggesting that protein synthesis is not inhibited by TGFβ in these cells (Fig. 7A). The level of SMC (ASGP-2) precursor is similar for treated and untreated samples, again suggesting that TGFβ does not inhibit the translation of SMC (ASGP-2) (Fig. 7A). To determine whether TGFβ 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 TGFβ, SMC precursor is processed rapidly into mature ASGP-2, such that >50% of the precursor is processed into mature ASGP-2 in 1 h (Fig. 7B). In the presence of TGFβ, the 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 TGFβ-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 TGFβ 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 TGFβ. Once again, these

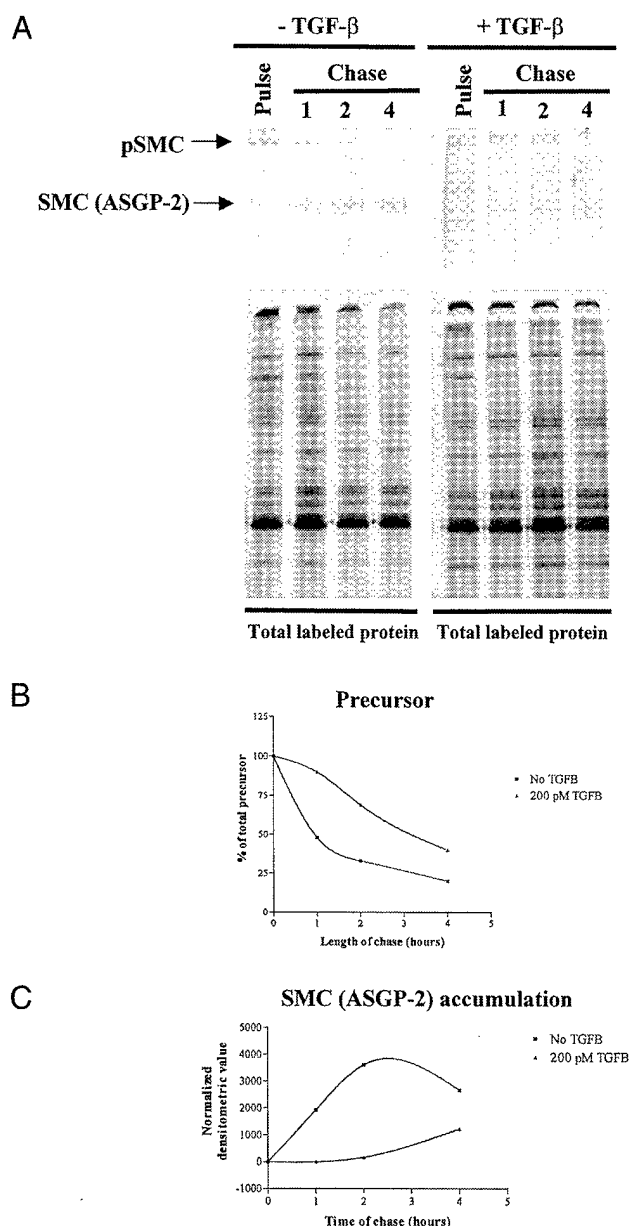


FIG. 7. Effect of TGF β on SMC precursor processing in normal cultured MEC. A, normal virgin MEC were isolated and cultured in the presence or absence of 200 pM TGF β . After 48 h cells were metabolically labeled with [^{35} S]Cys + [^{35} S]Met. After a 30-min pulse labeling, the medium was replaced with non-radioactive medium, and cells were harvested at various times, as indicated. Samples were immunoprecipitated, and immunoprecipitates and non-immunoprecipitated whole cell lysate samples were subjected to SDS-PAGE and fluorography. B, plot of SMC precursor processing into ASGP-2. The precursor bands from A were quantified by densitometry, and the results were plotted. C, plot of accumulation of mature ASGP-2. The mature ASGP-2 bands from A were quantified by densitometry, and the results were plotted.

data point to a different mechanism of post-transcriptional regulation of SMC from that with Matrigel, which occurs by a reduction in SMC precursor synthesis.

DISCUSSION

SMC is expressed in a number of normal rat tissues and is developmentally regulated in normal rat mammary gland. Without tight regulation, overexpression of this protein could have profound deleterious effects on the mammary epithelium, including disruption of cell-cell and cell-matrix interactions. To achieve this precise regulation, a complex series of regulatory

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 TGF β 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.

TGF β 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). TGF β 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 TGF β 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, TGF β 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 TGF β does not inhibit casein synthesis from lactating organ explant cultures or MEC from lactating mice. Taken together, these data suggest that TGF β 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 TGF β . 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 TGF β 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 TGF β (4, 6). 3) The mechanism of regulation appears to be post-transcriptional for both proteins. These data support a role for TGF β as an inhibitor of milk protein synthesis and accumulation in the virgin or pregnant mammary gland.

TGF β represses SMC (ASGP-2) levels in mammary epithelial cells whether or not the mammary epithelial cells are cycling. This result suggests that TGF β -induced cell cycle arrest and TGF β repression of SMC (ASGP-2) levels occur by different mechanisms (different signaling pathways). Administration of TGF β 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 TGF β is independent of the cell cycle and is not a result of growth inhibition. The repression of SMC (ASGP-2) expression by TGF β 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, TGF β 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 TGF β by disrupting SMC precursor processing. β -Casein mRNA is stabilized by the presence of prolactin (35), and its synthesis is inhibited by

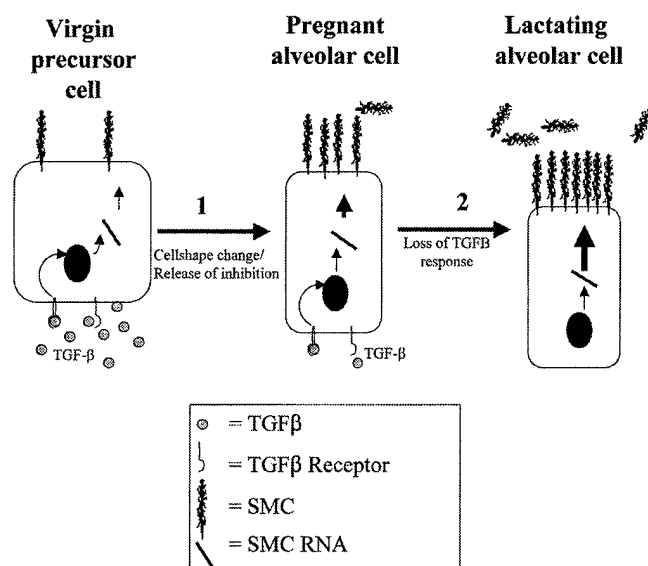


FIG. 8. Model for repression of SMC (ASGP-2) expression in normal mammary epithelial cells by extracellular matrix and TGF β .

TGF β (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).

TGF β has been implicated in a number of post-transcriptional regulatory mechanisms. TGF β can regulate gene expression post-transcriptionally by increasing or decreasing the stability of mRNAs. In osteoblast cell cultures TGF β can inhibit collagenase 3 expression by accelerating the decay of its transcript (38). In vascular smooth muscle cells TGF β can stabilize lysyl oxidase mRNA (39). Other mechanisms of post-transcriptional regulation by TGF β have also been proposed. For example, TGF β 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, TGF β induces higher secreted levels of collagenase MMP-2 by increasing the stability of the secreted 72-kDa proenzyme (41). TGF β 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 TGF β . 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) production by the presence of SMC (ASGP-2) transcript, whose expression is regulated by cell differentiation and insulin/insulin-like growth factor.³ Translation of this transcript is repressed by an inhibition related to cell environment, mimicked by Matrigel. High levels of TGF β in the virgin mammary gland further control SMC precursor by regulating its processing. As pregnancy proceeds the cell environment changes, and active TGF β levels decrease,⁴ allowing for increased translation and processing. Finally, at the onset of lactation TGF β levels be-

come undetectable, and SMC (ASGP-2) is translated and processed at a higher levels. Isolation of MEC causes disruption of the cell environment and loss of TGF β signaling, resulting in an overexpression of SMC (ASGP-2), which can be reversed by Matrigel and TGF β addition. Similarly, neoplastic transformation can lead to a loss of cell polarization and basement membrane contact, releasing the inhibition on precursor synthesis. Loss of TGF β responsiveness during tumor progression (42) will also release the post-translational processing block and lead to frank overexpression of SMC (ASGP-2), with its potential for deleterious consequences.

These studies raise other questions about regulation of SMC (ASGP-2) expression in normal mammary gland by TGF β . Two signaling pathways have been implicated in TGF β effects: the SMAD pathways (43) and the MAP kinase pathway (44). Preliminary experiments with MAP kinase pathway inhibitors suggest that SMC (ASGP-2) regulation by TGF β does not involve the MAP kinase pathway. Whether SMADs are involved is uncertain, and studies are currently under way to investigate this possibility. Another question is whether TGF β regulates casein and SMC (ASGP-2) by similar mechanisms. This seems unlikely because of the specificity of the effect on SMC (ASGP-2), occurring at a specific stage of SMC (ASGP-2) processing. One possible explanation for the TGF β effect on SMC (ASGP-2) is that it inhibits the enzyme that cleaves SMC (ASGP-2) precursor into ASGP-1 and ASGP-2. MUC2 has the same sequence, N-GDPH-C, at its putative cleavage site (24), suggesting that it may be cleaved (processed) by the same enzyme (or family of enzymes). Thus, if the cleavage enzyme is regulated by TGF β , this mechanism of regulation may be applicable to other mucins, though probably not to casein. However, the TGF β effect could also be due to a post-translational modification, such as glycosylation or phosphorylation, which could affect both SMC (ASGP-2) and casein processing and their subsequent behavior. Additional experiments are in progress to investigate these possibilities.

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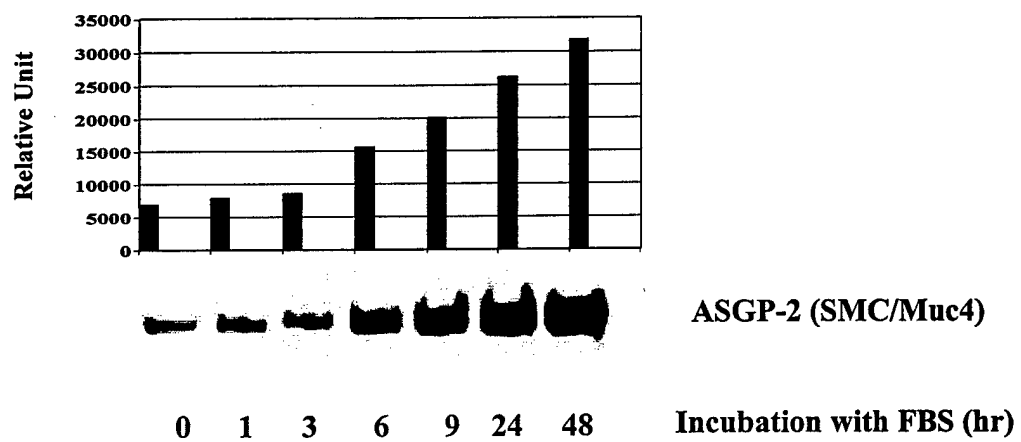
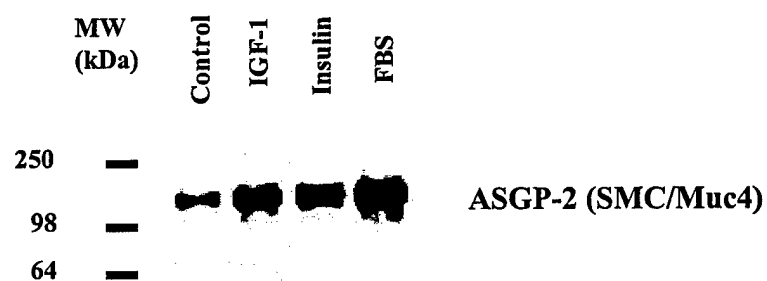


FIG. 1

A.



B.

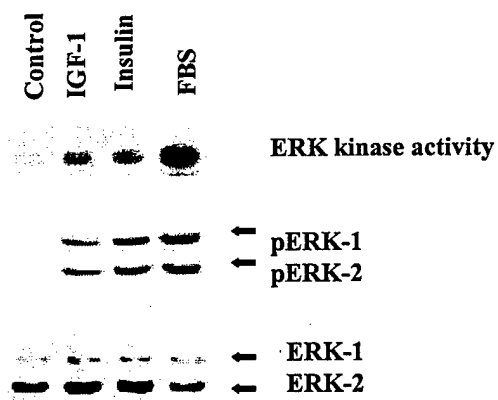
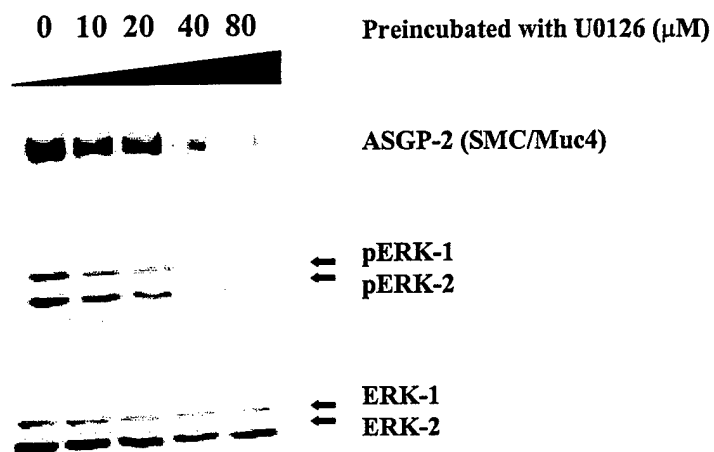


FIG. 2

A.



B.

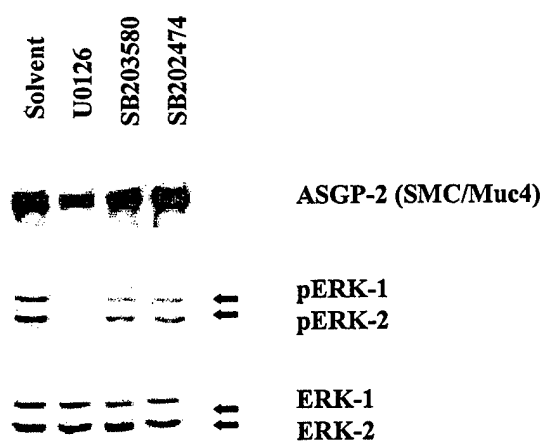


FIG. 3

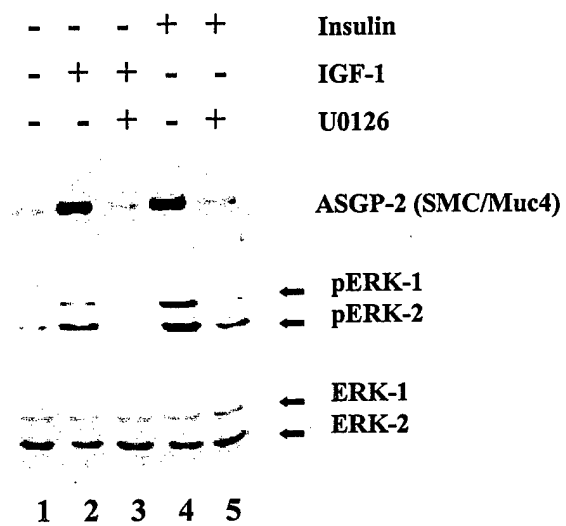


FIG. 4

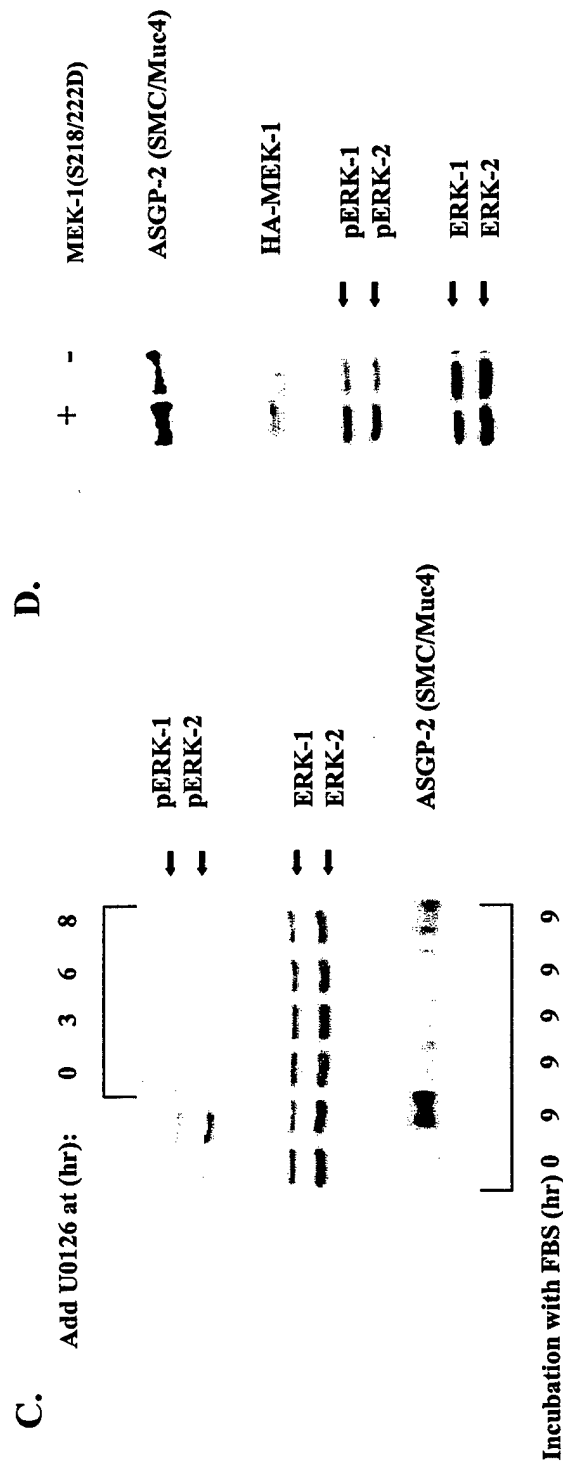
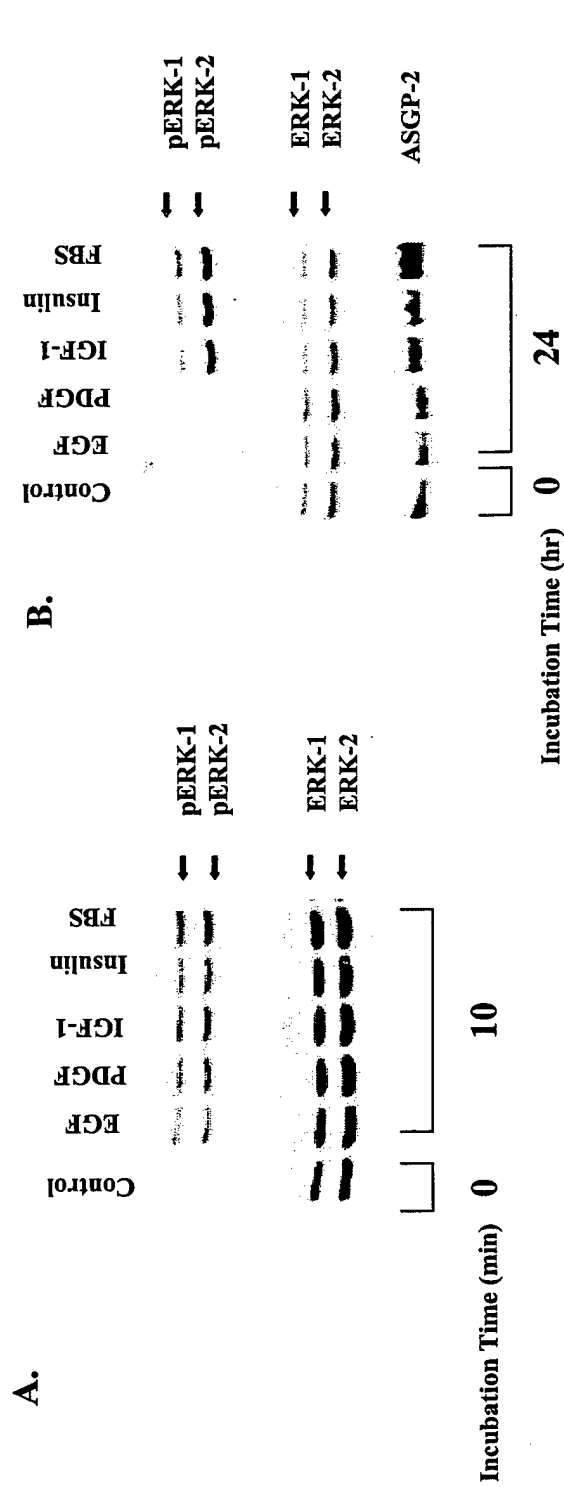


FIG. 5

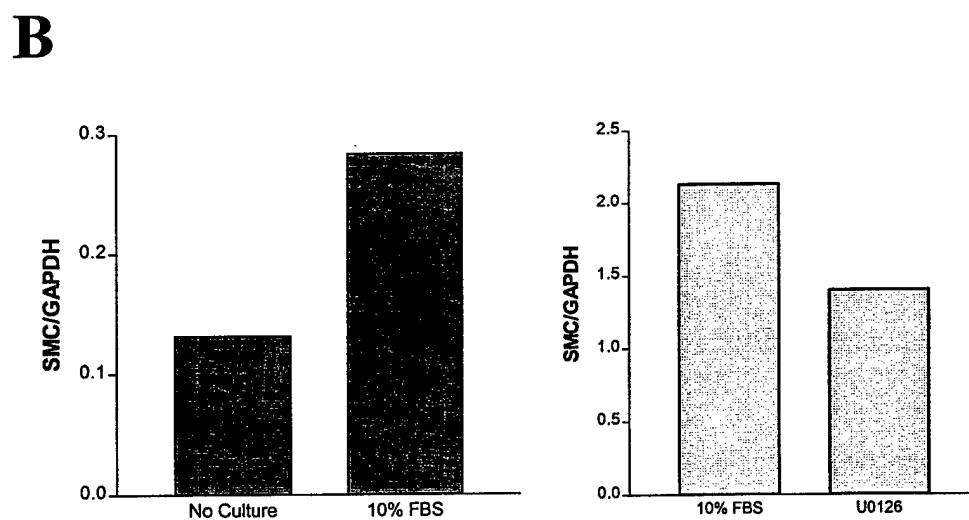
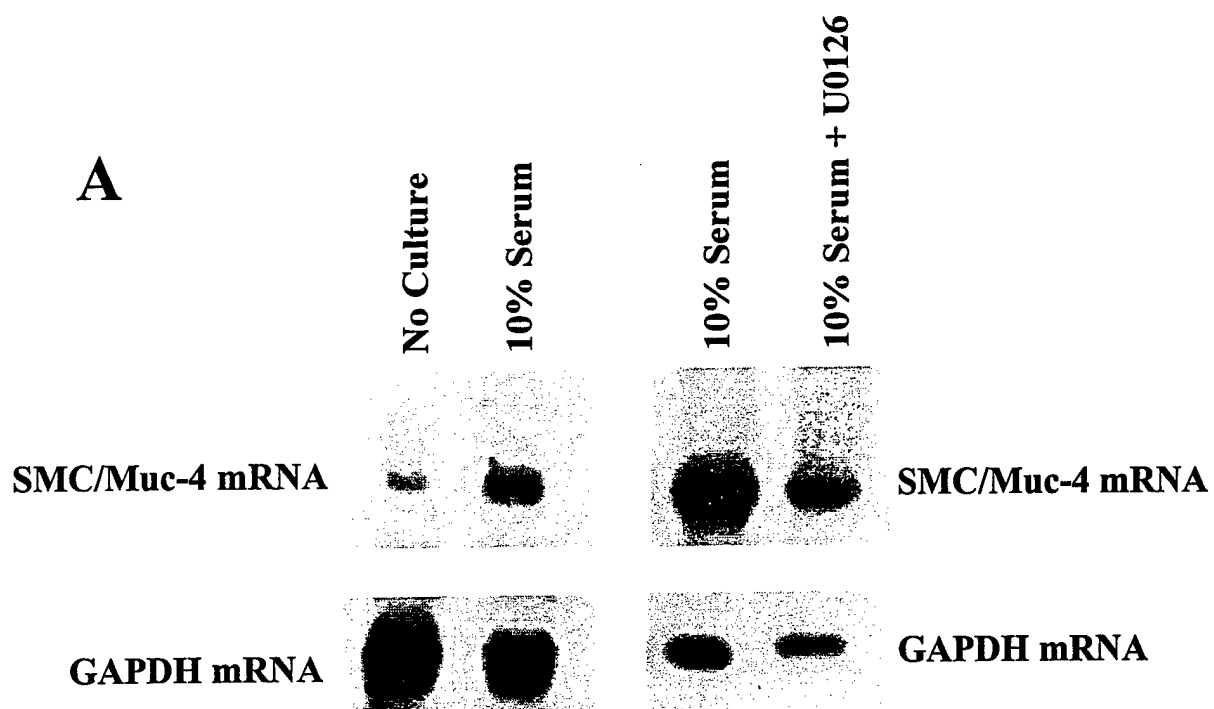


FIG. 6

Post-transcriptional Regulation of a Milk Membrane Protein, the Sialomucin Complex (Ascites Sialoglycoprotein (ASGP)-1/ASGP-2, Rat Muc4), by Transforming Growth Factor β *

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Sialomucin complex (SMC, Rat Muc4) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 (ascites sialoglycoprotein-1) and a transmembrane subunit ASGP-2, which can act as a ligand for the receptor tyrosine kinase ErbB2. SMC is highly expressed on the surface of ascites 13762 rat mammary adenocarcinoma cells, approximately 100 times the level in lactating mammary gland and 10^4 times that in virgin mammary gland. SMC is sharply increased at mid-pregnancy in a manner similar to β -casein. Unlike β -casein, SMC appears to be regulated post-transcriptionally. Its transcript is present in both virgin and pregnant mammary tissue, and SMC synthesis is induced rapidly in cultured primary mammary epithelial cells from either normal pregnant or virgin rats. SMC protein, but not transcript, levels are significantly reduced when mammary cells are cultured in Matrigel, a reconstituted basement membrane which stimulates casein expression. SMC precursor is synthesized in Matrigel at a 10-fold lower rate. Matrigel has no effect on either the level of SMC or its transcript in cultured 13762 mammary tumor cells. The Matrigel effect on primary mammary and 13762 cells is mimicked by transforming growth factor β , a component associated with this complex matrix. These results indicate that SMC is a novel product of normal mammary gland and milk, which is post-transcriptionally regulated by transforming growth factor β in normal mammary gland, but not in 13762 mammary adenocarcinoma cells.

Mammary gland development and differentiation are regulated by complex interactions of growth factors, hormones, and ECM¹ (1–5). The regulation of some mammary specific milk proteins by ECM has been studied in detail. For example, β -casein, an “early” milk protein which is detectable around day 6 of pregnancy in the mouse, is transcriptionally regulated by laminin through β 1 integrins (6, 7). Whey acidic protein

(WAP), a “late” milk protein whose message becomes detectable around day 14 of pregnancy in the mouse, is dependent on an ECM-induced three-dimensional alveolar structure (6). When normal mammary epithelial cells (MEC) are placed in culture, WAP transcription is unaffected by the presence or absence of basement membrane, suggesting an additional post-transcriptional regulatory mechanism (6). Thus, both the combinations of factors necessary for proper regulation of expression of milk protein genes and the mechanisms of regulation are complex.

SMC, which was recently identified as one of the milk membrane mucins (8), was originally discovered as the major glycoprotein complex on the surface of highly malignant, metastatic 13762 rat ascites mammary adenocarcinoma cells (9, 10). The complex consists of an O-glycosylated mucin subunit ASGP-1 (9–12), which is tightly, but non-covalently, bound to an N-glycosylated integral membrane glycoprotein ASGP-2 (10, 13). SMC is transcribed from a single gene as a 9-kilobase transcript (14, 15), which is translated into a single polypeptide which is proteolytically cleaved early in its transit to the cell surface (16). Mature glycosylated ASGP-1 has a molecular mass of >500 kDa (9), with a polypeptide molecular mass of $\approx$ 220 kDa (15), and comprises three domains: an N-terminal unique sequence, a large tandem repeat region rich in serine and threonine residues similar to that of other mucins, and a C-terminal unique sequence (15). ASGP-2 is a 120–140-kDa protein that consists of seven domains: two hydrophilic N-glycosylated regions, two EGF-like domains, a cysteine-rich domain, a transmembrane domain, and a small cytoplasmic domain (14).

In tumor cells sialomucin complex may have dual functions.

1) ASGP-1 can provide anti-recognition and anti-adhesive properties to tumor cells (11, 12, 18). In A375 melanoma cells stably transfected with SMC DNA linked to a tetracycline-regulated promoter, expression of SMC abolishes cell-matrix adhesion and cell-cell interactions (18). Furthermore, SMC expression in these cells reduces their killing by natural killer cells.² This anti-recognition property may be important to the high metastatic capacity of the 13762 ascites cells (9, 11, 19). 2) The two EGF-like domains have all of the consensus residues present in active members of the EGF growth factor family (14), and SMC has been shown to bind to the receptor ErbB2. Thus, the transmembrane subunit ASGP-2 is proposed to modulate signaling through the EGF family of receptors via its interaction with ErbB2,³ the critical receptor for formation of

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¹ The abbreviations used are: ECM, extracellular matrix; WAP, whey acidic protein; SMC, sialomucin complex; ASGP, ascites sialoglycoprotein; EGF, epidermal growth factor; MEC, mammary epithelial cells; PBS, Dulbecco's phosphate-buffered saline without calcium; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; TGF β , transforming growth factor β .

² M. Komatsu, C. A. C. Carraway, N. Fregien, and K. L. Carraway, manuscript in preparation.

³ K. L. Carraway III, E. A. Rossi, S. A. Price-Schiavi, M. Komatsu, D. Huang, P. M. Guy, M. E. Carvajal, N. Fregien, C. A. C. Carraway, and K. L. Carraway, submitted for publication.

active heterodimeric class I receptor tyrosine kinases (20), for which no ligand has been described to date. This interaction may play a role in the constitutive phosphorylation of ErbB2 in the 13762 ascites cells (21) and the high proliferative activity of these cells.

Sialomucin complex is not mammary specific. It is expressed in a number of normal secretory epithelial tissues in the adult rat, including small and large intestine, trachea, and uterus (8, 17). In the trachea it is constitutively expressed on the apical surface of the cells lining the lumen (17). SMC is also constitutively expressed in the normal adult rat intestine. In the adult small intestine SMC is observed only in Paneth cells at the bases of the crypts of Lieberkuhn, while in the colon it is found specifically in goblet cells (8). In the uterus, SMC is observed on the luminal surface of the endometrium and in glandular cells. Its expression is regulated at the mRNA level by the ovarian hormones estrogen and progesterone (22). These results suggest that SMC has multiple and complex regulatory mechanisms in the normal adult rat.

SMC is abundant in milk (ASGP-2 concentration, $\sim 10 \mu\text{g/ml}$) and lactating mammary gland, but its level is very low in the virgin gland. In lactating mammary tissue it is localized at the apical surface of secretory epithelial cells lining the alveoli. It appears to be located within the cytoplasm, not limited to the plasma membrane or milk fat globule membrane, suggesting that its location is in the mammary secretory granules (8). In this tissue SMC is expressed as both membrane and soluble forms, the soluble form lacking the transmembrane and C-terminal domains. Interestingly, the level of SMC in the 13762 ascites cells is about 100-fold greater than that in the lactating mammary gland (8). These results suggest that SMC is developmentally regulated in normal mammary gland, but that this regulation is lost in the rat mammary ascites tumor.

Recent studies have helped to define the place of SMC among the hierarchy of known mucins. Cloning and sequencing of full-length human MUC4 show substantial similarities between the MUC4 and rat SMC at the N- and C-terminal portions of the molecules (23).⁴ They differ in their repeat domains. In particular, SMC does not have the 16-amino acid repeat cloned and sequenced in the original description of MUC4 (24). Thus, the similarity between the molecules was not observed previously. However, the 70% identity between the human MUC4 analog of ASGP-2 and rat ASGP-2 provides strong evidence that they are homologous proteins.

In an effort to understand the regulation of SMC in normal mammary gland, we have examined both mammary tissue and cultures of primary mammary epithelial cells. *In vivo* SMC expression is superficially similar to that of β -casein and is clearly developmentally regulated. *In vitro*, however, in contrast to β -casein, which requires ECM for expression, SMC levels are significantly reduced in the presence of Matrigel, a reconstituted ECM preparation. Unlike β -casein, high levels of SMC can be induced within hours in cultured virgin mammary epithelial cells. Furthermore, SMC message is expressed at all times in the normal developing mammary gland, unlike the protein, which is not present in virgin gland and increases as pregnancy proceeds. In addition, TGF β can reduce the level of SMC protein without affecting the level of SMC transcript, suggesting that it is the factor responsible for post-transcriptional regulation of SMC in normal MEC. These data suggest that SMC is a novel post-transcriptionally regulated product of the mammary gland and milk.

EXPERIMENTAL PROCEDURES

Materials—The MAT-B1 ascites subline of the 13762 rat mammary adenocarcinoma was maintained by weekly passage, as described previously (25). Anti-ASGP-2 polyclonal antiserum used for immunoprecipitations (16) and mouse monoclonal antibody 4F12 used for immunoblots (8) have been described previously. Anti- β -casein mouse monoclonal antibody used for immunoblots was kindly provided by Dr. Charlotte Kaetzel (University of Kentucky, Lexington, KY). TGF β was purchased from R & D Systems, Inc. Cell culture materials were obtained from Life Technologies, Inc., unless otherwise noted.

Blotting Procedures—For Northern blots total RNA was isolated from whole normal mammary tissue, mammary epithelial cells, or 13762 MAT-B1 cells using TRI REAGENT™ (Molecular Research Center, Inc., Cincinnati, OH), and 25 μg were electrophoresed on 1% formaldehyde/agarose gels. Resolved RNAs were transferred to Zeta-Probe positively charged nylon membranes (Bio-Rad), followed by cross-linking using a Stratalinker (Stratagene, La Jolla, CA). The membranes were prehybridized for at least 2 h at 42 °C in prehybridization solution (50% formamide, 5 $\times$ SSC, 5 $\times$ Denhardt's reagent, 0.1% SDS, and 0.5 mg/ml salmon sperm DNA). The probe, A2G2-9, a 1.7-kilobase probe which spans the 5' unique region and four tandem repeats of SMC cDNA, was random primed labeled with [³²P]dCTP using a Random Primed Labeling kit (Boehringer-Mannheim). The membranes were hybridized overnight at 42 °C in prehybridization solution containing 0.1 g/ml dextran sulfate and the labeled probe. Following hybridization, membranes were washed once at room temperature in 2 $\times$ SSC with 0.1% SDS for 15 min, twice at 50 °C in 2 $\times$ SSC with 0.1% SDS for 20 min each, and once at 50 °C in 0.1X SSC with 0.1% SDS for 15 min. Signals were detected by exposure with Kodak XAR-5 x-ray film.

For Western blots, SDS-PAGE was performed under reducing conditions using 6% polyacrylamide gels and the mini-Protein II system (Bio-Rad). Resolved proteins were transferred to nitrocellulose membranes which were subsequently blocked with 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, Rockford, IL) diluted 1:20,000 in 1% bovine serum albumin/Tris-buffered saline with 0.5% Tween 20. Signals were detected with the Renaissance™ Enhanced Chemiluminescence kit (NEN Life Science Products Inc., Boston, MA).

Preparation of Tissue Samples—Mammary tissue dissected from female Fischer 344 rats was pulverized with a mortar and pestle in liquid N₂ and stored as a powder at -80 °C. For immunoblotting, tissue powders were solubilized directly into SDS-PAGE sample buffer at a concentration of 10 mg/ml wet weight. For quantitation of total protein, powders were solubilized in 1% SDS in water, boiled, and clarified by centrifugation at 12,000 $\times$ g. Protein concentrations were determined by Lowry assay of the cleared SDS lysates.

Preparation of Mammary Epithelial Cells—Primary mammary epithelial cell cultures were established using previously described protocols (26–29). Briefly, mammary glands excised from virgin or pregnant female Fischer 344 rats were minced, resuspended in digestion media comprised of 1 mg/ml collagenase type II (Worthington Biochemical Corp., Freehold, NJ), and 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin in Ham's F-12 medium (Life Technologies, Inc.) and incubated at 37 °C with shaking for 45 min. Fully and partially digested epithelial cell clusters were pelleted and incubated a second time in digestion buffer at 37 °C with shaking for 45 min. Digested epithelial cell clusters were pelleted, resuspended in PBS, and passed through a 520- μm cell sieve to remove undigested material. Mammary epithelial cell clusters in the resulting filtrate were captured on a 70- μm nylon membrane. Cell clusters were collected by rinsing the membrane with PBS and were subsequently washed three times in PBS prior to plating. Incubating freshly isolated cells on a plastic tissue culture plate for 1 h permitted attachment and removal of fibroblasts.

Cell Culture and Analysis—Mammary epithelial cell clusters were resuspended and plated in equal aliquots in Ham's F-12 medium containing 1 mg/ml bovine serum albumin (Sigma) and either 10% FCS and 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin or 5 $\mu\text{g/ml}$ insulin, 10 $\mu\text{g/ml}$ transferrin, 0.3 $\mu\text{g/ml}$ sodium selenite, and 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin. Where indicated, media were supplemented with 5 $\mu\text{g/ml}$ insulin, 1 $\mu\text{g/ml}$ hydrocortisone, 3 $\mu\text{g/ml}$ prolactin, and/or various concentrations (2.5–20 ng/ml) of TGF β . For plating with embedding in ECM (Matrigel, Collaborative Biomedical Products, Bedford MA), cells were resuspended in 1.5 ml of ice-cold Matrigel diluted

⁴ J.-P. Aubert, results presented at the Fifth Workshop on Tumor Mucins, Cambridge, United Kingdom.

1:3 with serum-free Ham's F-12 medium, plated at 1.5 μ /mm² of tissue culture plastic, and allowed to solidify at 37 °C for 30 min. The solidified Matrigel was then overlaid with 2 ml of either serum-free or serum-containing medium. Cells were cultured at 37 °C in 5% CO₂ for 48 h prior to harvest. Cells were collected from culture on plastic dishes by scraping cells off the dish. Cells were harvested from Matrigel cultures using the recommended protocol for Matrisperse (Collaborative Biomedical Products, Bedford, MA), an enzyme-free Matrigel dissociation buffer. For all samples, harvested cells were pelleted, washed with PBS, and lysed in 100 μ l of 1% SDS in water. Protein concentration of the cell lysates was determined by Lowry assay, and 5 μ g of total protein was loaded for immunoblot analysis.

Labeling of Mammary Epithelial Cells—Mammary epithelial cells were isolated from virgin rats and cultured either on plastic or embedded in Matrigel in serum-free medium. After 24 h cells were washed twice with PBS, starved for 30 min in Cys/Met free Dulbecco's minimal essential medium supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, and 10 mM Hepes, and incubated in 1 ml of labeling medium (starvation medium + 230 μ Ci of [³⁵S]Cys + [³⁵S]Met per ml) (EXPRE³⁵S³⁵S Protein Labeling Mix, NEN Life Science Products) for times ranging from 5 to 180 min. Labeled cells were washed twice with PBS and lysed in 400 μ l of 2% SDS in H₂O. Lysed cells were boiled for 1 min, sonicated for 10 min in a bath sonicator, and diluted in 2 ml of Triton immunoprecipitation buffer (2.5% Triton X-100, 190 mM NaCl, 60 mM Tris-HCl, 6 mM EDTA, pH 7.4). Diluted cell lysates were centrifuged at 175,000 $\times$ g for 40 min at 4 °C. Cell lysates were immunoprecipitated with polyclonal anti-ASGP-2 antiserum and protein A-agarose beads (Sigma) overnight at 4 °C with rotation. Immunoprecipitates were washed with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris base, pH 8.0) six times for 15 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 20 μ l of SDS sample buffer and immunoprecipitate supernatant was diluted 1:1 in SDS sample buffer. Diluted samples were analyzed by SDS-PAGE and fluorography with Fluoro-Hance autoradiography enhancer (Research Products International Corp., Mount Prospect, IL).

RESULTS

Time Course of SMC Expression in Normal Developing Mammary Gland—Mammary homogenates from virgin, pregnant, lactating, and involuting rats were analyzed by anti-ASGP-2 and anti- β -casein immunoblots to study the expression pattern of SMC in normal developing mammary gland and to compare its expression pattern to that of β -casein. 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 antibodies to ASGP-2 are more sensitive and more specific than those for ASGP-1. As previously reported (8), SMC is minimal in virgin mammary gland. Its level increases sharply (approximately 40-fold) during mid-pregnancy, reaches a maximum in late pregnancy and during lactation, and decreases during involution (Figs. 1, A and B). The expression pattern of β -casein is similar, but it reaches maximal levels about 1 to 2 days earlier than SMC. β -Casein is maintained throughout lactation and decreases more rapidly than SMC during involution. These data suggest that, like β -casein, SMC is developmentally regulated in normal mammary gland, but that it may have a different regulatory mechanism.

The mammary gland is made up of several cell types. SMC is expressed in the secretory luminal epithelial cells (8). Thus, it is necessary to study the level of SMC in isolated MEC to determine if the apparent increase in SMC in developing mammary gland is a consequence of a real increase in SMC production or an increase in the proportion of epithelial cells relative to other cell types during pregnancy. For this reason mammary epithelial cells were isolated by collagenase digestion from virgin and pregnant rats, and anti-ASGP-2 and anti- β -casein immunoblots were performed. To test whether cell surface SMC is degraded by collagenase, MAT-B1 ascites cells were treated with collagenase under the same conditions. Immuno-

blots of collagenase-treated ascites cells showed no difference in the level of SMC compared with that of untreated cells (data not shown). As seen in whole tissue, SMC is low in virgin mammary epithelial cells and increases sharply in MEC from mid- and late-pregnant animals (Fig. 1C). β -Casein expression is also minimal in virgin MEC and is detectable at maximum levels in MEC from both mid- and late-pregnant animals. As seen in whole tissue, β -casein reaches maximum levels earlier than SMC. Thus, the expression of these proteins in the isolated cells reflects that in the mammary tissue and thus can be used as a model for the study of regulatory mechanisms.

To determine whether the expression pattern of the SMC transcript is similar to that for the protein, total RNA was prepared from mammary epithelial cells freshly isolated from virgin and pregnant rats, and Northern blot analysis was performed using probe A2G2-9, which was generated from the 5' 1.7-kilobases of SMC cDNA. Surprisingly, SMC transcript is present at equivalent levels in MEC at all time points tested (Fig. 2). This expression pattern for SMC transcript is different from that for the protein, which is low in virgin gland and increases sharply as pregnancy proceeds (Fig. 1). Furthermore, this pattern also differs from that of β -casein message, which is undetectable in MEC until mid-pregnancy (6). These results suggest that, unlike β -casein, regulation of SMC in normal rat mammary epithelial cells *in vivo* occurs post-transcriptionally.

Expression of SMC Is Regulated by Matrigel—Primary culture of mammary epithelial cells has been used to elucidate the regulatory mechanisms of several milk proteins, including β -casein. These systems are useful because the level of differentiation/functionality can be maintained and manipulated. In the presence of ECM and lactogenic hormones (insulin, hydrocortisone, and prolactin), isolated mammary epithelial cells will form round alveolar structures with closed lumens and will vectorially secrete milk proteins. On plastic these cells form squamous epithelial monolayers and are considered undifferentiated because milk proteins are not synthesized. The *in vivo* expression patterns of SMC and β -casein are different, suggesting that SMC may be regulated differently than β -casein or other early milk protein genes. Expression of β -casein in isolated mammary epithelial cells is dependent on the presence of extracellular matrix components (or basement membrane). In the presence of a reconstituted basement membrane (Matrigel), mammary epithelial cells from pregnant mice and virgin rats can be induced to express β -casein. To investigate the effect of extracellular matrix on SMC, mammary epithelial cells were isolated from mid-pregnant (day 11) rats, when SMC is low and casein is high. Cells were cultured either on plastic or embedded in Matrigel in the presence or absence of FCS. After 48 h, cleared cell lysates were prepared for anti-ASGP-2 and anti- β -casein immunoblots, and total RNA was prepared for Northern blot analysis with probe A2G2-9. In freshly isolated mid-pregnant mammary epithelial cells the level of SMC is undetectable, while that of β -casein is high (Fig. 3A). However, when the MEC are cultured on plastic with 10% fetal calf serum, SMC is detected at a high level, while β -casein, as expected, is reduced significantly. In the absence of serum on plastic SMC is expressed at a lower level, and β -casein is undetectable. In the presence of Matrigel and serum the level of SMC is low and that of β -casein is high. In Matrigel without serum the level of SMC is minimal and β -casein is unaffected. These data suggest that SMC levels are significantly enhanced by a factor in FCS, but greatly reduced by a factor present in the reconstituted basement membrane. Interestingly, although the protein level of SMC is negatively affected by the presence of Matrigel, the SMC transcript level is not decreased by culture on plastic or in Matrigel (Fig. 3B). The negative effect of Matrigel on SMC

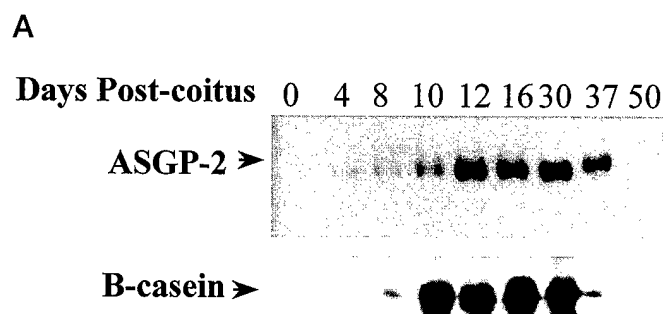
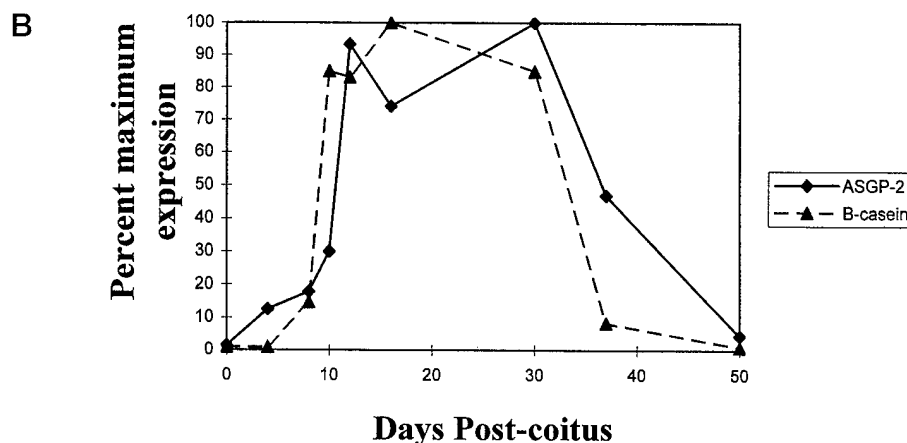
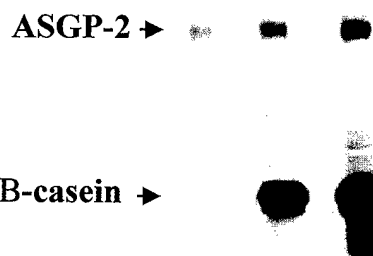


FIG. 1. Expression of SMC and β -casein in the mammary gland and epithelial cells. SDS-solubilized samples of virgin (day 0), pregnant (post-coitus days 4, 8, 10, 12, and 16), lactating (day 30), and involuting (days 37 and 50) mammary tissue were loaded (50 μ g wet weight/lane) for SDS-PAGE as indicated at the top of each blot. A, immunoblots with anti-ASGP-2 or anti- β -casein antibodies are as indicated at the left of the figure. B, plot of percent maximum expression during mammary development. The bands from A were quantified by densitometry and the sample with the most intense staining was used as 100% (day 30 for SMC and day 16 for β -casein). C, SDS-solubilized samples of isolated mammary epithelial cells from virgin (day 0) and pregnant (days 11 and 18) rats were loaded (5 μ g/lane total protein) for SDS-PAGE as indicated at the top of each blot. Immunoblots were performed with anti-ASGP-2 or anti- β -casein antibodies as indicated at the left of the figure.



C Days Post-coitus 0 11 18



Days Post-coitus 0 11 18

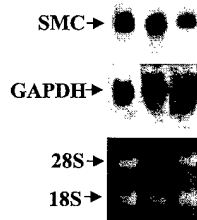


FIG. 2. Northern blot analysis of SMC mRNA in normal rat mammary epithelial cells. Total RNA was isolated from virgin (day 0) and pregnant (days 11 and 18) mammary epithelial cells, and 25 μ g/lane were loaded for Northern blot analysis. Northern blots were probed with A2G2-9 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as indicated at the left of the figure.

protein levels is very different from its effect on β -casein, which is regulated by the ECM from the transcript level. These results suggest that SMC, under these conditions, is regulated post-transcriptionally, as it appears to be *in vivo*. Thus, this culture system is a useful *in vitro* model for determining factors that affect SMC expression in normal mammary gland.

SMC in MEC from Virgin Animals— β -Casein expression requires pregnancy or MEC culture under specific conditions that mimic the pregnant state (29, 30). Because the SMC transcript is present in abundance in MEC from virgin animals, these cells may already be primed for SMC production and awaiting the appropriate signal to synthesize the protein. Mammary epithelial cells were isolated from virgin rats and cultured on plastic with 10% serum to determine whether priming by pregnancy is necessary for the induction of SMC protein production or whether a change in the environment is sufficient for SMC biosynthesis. After 24 to 72 h in culture, the cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 antibodies. In cultured virgin mammary epithelial cells, SMC reaches a maximal level in 24 h, and the level is maintained for at least 48 h (Fig. 4A). Thus, pregnancy is not required for the production of SMC. Induction of SMC biosynthesis appears to require only release of mammary epithelial cells from their environment, suggesting that in the virgin gland there is an inhibition of SMC synthesis which is released by removal of MEC or by the changes induced by pregnancy.

To determine whether extracellular matrix affects SMC in MEC from virgin animals similarly to that for MEC from preg-

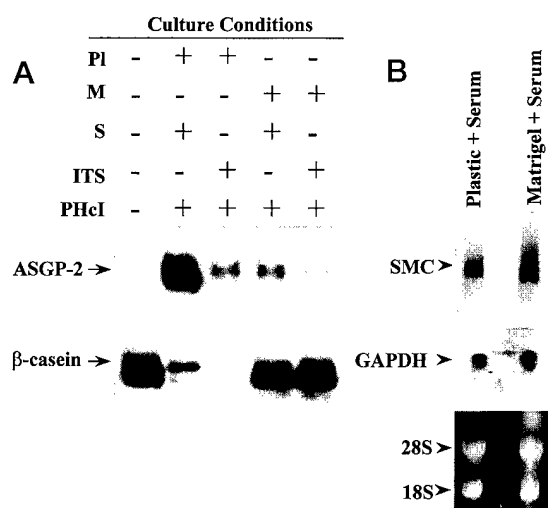


FIG. 3. Effect of Matrigel on SMC expression in pregnant rat mammary epithelial cells. Normal rat mammary epithelial cells were collected by collagenase digestion of day 11 pregnant rat mammary tissue followed by differential centrifugation. MEC were plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence or absence of 10% FCS as indicated at the top of the figure. **A**, immunoblots of cultured pregnant mammary epithelial cells. SDS-solubilized cell lysates were loaded (5 μ g/lane) for SDS-PAGE, and immunoblots were performed with monoclonal antibodies against ASGP-2 and β -casein as indicated at the left of the figure. **B**, Northern blot of cultured pregnant mammary epithelial cells. Total RNA was isolated from cultured epithelial cells and 25 μ g/lane was loaded for Northern blot analysis. Northern blots were probed with A2G2-9. *Pl*, plastic; *M*, Matrigel; *S*, serum; *ITS*, insulin, transferrin, and selenium; *PHcI*, lactogenic hormones prolactin, hydrocortisone, and insulin.



FIG. 4. Expression of SMC in mammary epithelial cells from virgin rats. Normal rat mammary epithelial cells were collected by collagenase digestion of virgin rat mammary tissue. MEC were plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence of 10% FCS. Immunoblots were performed with anti-ASGP-2 antibodies as indicated at the left of the figure. **A**, induction of SMC expression in cultured virgin mammary epithelial cells. **B**, effect of Matrigel on SMC expression in cultured mammary epithelial cells from virgin rats.

nant animals, MEC from virgin rats were isolated and cultured in the presence of 10% serum either on plastic or embedded in Matrigel as described above. After 48 h in culture, cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 antibodies. In freshly isolated mammary epithelial cells, SMC is undetectable, as expected. When cultured on plastic, the level of SMC is high, and as in the cultured MEC from pregnant rats, the presence of Matrigel greatly inhibits the production of SMC (Fig. 4B). This inhibition occurs both when the cells are embedded in the Matrigel and when they are plated on top of it (data not shown), suggesting that one or more factors present in the Matrigel is involved in the reduction of

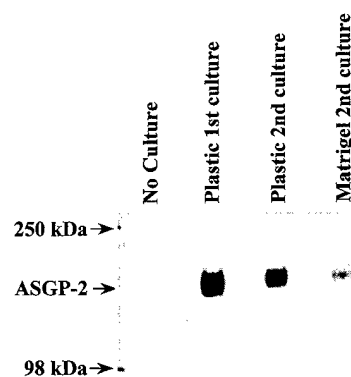


FIG. 5. Effect of Matrigel on SMC levels in normal mammary epithelial cells already expressing SMC. Virgin MEC were isolated and cultured on plastic in Ham's F-12 medium supplemented with 10% FCS. After 48 h, cells were removed from the plate with a non-enzymatic cell dissociation buffer. Half the cells were then replated in Ham's F-12 medium supplemented with 10% FCS either on plastic or embedded in Matrigel. After an additional 48 h in culture, cells were harvested and 5 μ g total protein was subjected to immunoblot analysis with anti-ASGP-2 antibodies.

SMC levels in cultured mammary epithelial cells.

To study the effect of Matrigel on SMC in normal MEC already expressing the protein, MEC from virgin rats were prepared and cultured on plastic with 10% serum to induce high levels of SMC production. After 48 h, the cells were removed from the plate by non-enzymatic cell dissociation buffer and replated either on plastic or embedded in Matrigel for 48 h. Cleared cell lysates were subjected to immunoblot analysis with anti-ASGP-2 antibodies. After 48 h on plastic, SMC was present in abundance (Fig. 5). When cells were replated on plastic, the level of SMC remained high but at a slightly lower level than that of the first plating. The level of SMC in the cells embedded in Matrigel was significantly reduced. Thus, the level of SMC in normal MEC which already express the protein can be modulated by Matrigel.

Effect of Matrigel on SMC Levels in MAT-B1 Tumor Cells—Matrigel contains a number of components, including proteases which could digest cell surface proteins, thereby decreasing the levels of SMC in MEC cultured in it. To test this possibility, MAT-B1 ascites tumor cells, which express SMC abundantly on the cell surface, were cultured on plastic or embedded in Matrigel for 48 h. Cleared cell lysates and total RNA were prepared and subjected to immunoblot analysis with anti-ASGP-2 antibodies and Northern blot analysis with probe A2G2-9, respectively. Whether cultured on plastic or in Matrigel, the level of SMC was unchanged in the cultured MAT-B1 tumor cells (Fig. 6A), suggesting that proteolysis does not cause the decreased level of SMC in Matrigel. This finding is consistent with the observation that the level of ErbB2 in MEC cultured in Matrigel is unaffected (data not shown). Moreover, culture of rat tracheal epithelial cells (31) in Matrigel does not reduce their level of SMC, as would be expected from a proteolytic effect at the cell surface. The level of SMC message in the ascites tumor cells was also unaffected by the presence or absence of Matrigel (Fig. 6B). These data suggest that cell surface SMC is not degraded by proteases in Matrigel, and that regulation of SMC by Matrigel has apparently been altered significantly in these tumor cells.

SMC Synthesis in Virgin MEC—Regulation of SMC by Matrigel appears to be post-transcriptional in cultured MEC. Formally, this type of regulation may be by modification or degradation of SMC message, modulation of SMC message translation, or degradation of SMC protein. The steady state

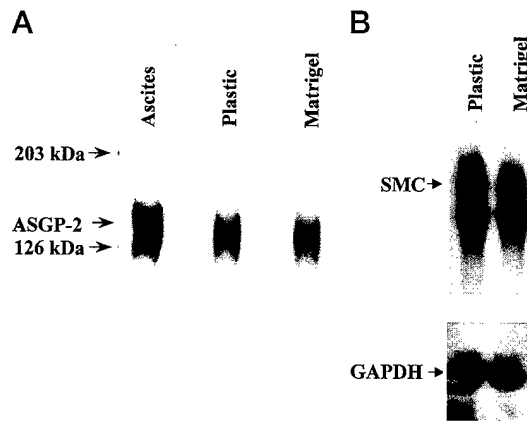


FIG. 6. Effect of Matrigel on expression of SMC in 13762 MAT-B1 tumor cells. Ascites MAT-B1 mammary adenocarcinoma cells were collected and plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence of 10% FCS. A, immunoblot with anti-ASGP-2 antibody. B, Northern blot of SMC message using probe A2G2-9 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

levels of SMC message are unaffected by different culture conditions, suggesting that SMC message degradation is not the mechanism by which SMC is regulated in this system. To study how Matrigel affects SMC message translation, mammary epithelial cells were cultured either on plastic or embedded in Matrigel. After 24 h, the cells were metabolically labeled with [^{35} S]Cys + [^{35} S]Met for times ranging from 5 to 180 min. After labeling, the cells were washed, lysed, and immunoprecipitated with polyclonal anti-ASGP-2 antibody, which recognizes the SMC precursor as well as mature ASGP-2. It was necessary to immunoprecipitate the precursor because this product is intracellular and would not be affected by an extracellular protease. Immunoprecipitated SMC precursor was subjected to SDS-PAGE and fluorography, as was an aliquot of immunoprecipitated supernatant. To normalize for total protein, the bands for the SMC precursor and total protein were quantified by densitometry. The value for the SMC precursor was compared with that obtained for total protein. The SMC precursor band from cells cultured on plastic was easily visible by 20 min of labeling, while that from cells cultured in Matrigel was not visible until 90 min of labeling (Fig. 7A). When the ratio of SMC precursor to total labeled protein was calculated, the cells cultured on plastic synthesized SMC about eight times faster than those cultured in Matrigel (Fig. 7B). Thus, a factor in Matrigel reduces the mammary epithelial cells' ability to synthesize SMC, suggesting that SMC is regulated at the translational level in this system.

Effect of TGF β on SMC Levels in Virgin MEC—Post-transcriptional regulation of milk protein gene expression and synthesis has not been widely explored. However, Robinson *et al.* (32) reported that TGF β treatment can suppress the ability of pregnant mouse mammary tissue explants to synthesize and secrete casein. TGF β treatment in their studies does not affect the level of casein mRNA, suggesting that it may regulate casein expression (synthesis) post-transcriptionally (32). TGF β is also a component of Matrigel, and may therefore be a factor responsible for the decreased synthesis of SMC when MEC are cultured in it. To investigate the effect of TGF β on SMC in normal virgin rat mammary gland, MEC were cultured in the presence or absence of 10% fetal bovine serum and 5 ng/ml TGF β either on plastic or embedded in Matrigel. After 48 h the cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 monoclonal antibodies. MEC cultured on plastic or in Matrigel with serum have higher levels of SMC than those cultured in serum-free conditions, as shown previ-

ously. Also as expected, MEC cultured in Matrigel have lower levels of SMC than those cultured on plastic. However, the addition of TGF β to MEC cultured in any of these conditions significantly reduces the level of SMC (Fig. 8), suggesting that TGF β may be the factor in Matrigel involved in decreasing the levels of SMC when MEC are embedded in it.

Dose Response of SMC Suppression by TGF β in Virgin MEC—The dose response of TGF β -induced suppression of SMC synthesis was studied in normal virgin MEC. The MEC were isolated and cultured on plastic under serum-free conditions in the presence of increasing doses of TGF β (0–20 ng/ml final concentration). After 48 h of culture, the cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 monoclonal antibodies. Maximum levels of SMC are present when no TGF β is added to the media, and as the level of TGF β increases, the level of SMC decreases (Fig. 9). Thus, suppression of SMC synthesis by TGF β in normal rat MEC is concentration-dependent. Furthermore, this response occurs at levels considered to be physiological.

Effect of TGF β on SMC Transcript Levels in Virgin MEC—TGF β suppresses the level of SMC in normal rat MEC in a manner similar to that of Matrigel. However, when SMC synthesis is suppressed by Matrigel, the level of SMC transcript is unaffected, suggesting a post-transcriptional mechanism of regulation in this system. To study the effect of TGF β on SMC transcript levels in the normal rat, MEC were isolated and cultured on plastic in Ham's F-12 medium supplemented with insulin, transferrin, and selenium and 10 ng/ml TGF β . After 48 h, the cells were harvested, total RNA was isolated, and 25 μ g of total RNA was subjected to Northern blot analysis with probe A2G2-9. Interestingly, while the level of SMC protein is significantly reduced by the presence of TGF β , the level of SMC transcript was relatively unaffected by the presence of TGF β under these conditions (Fig. 10). These results support a role for TGF β in the post-transcriptional regulation of SMC expression in normal rat mammary tissue.

DISCUSSION

SMC is developmentally regulated in normal rat mammary gland. *In vivo*, the protein is present at a low level in virgin gland and increases dramatically during mid-pregnancy, as does β -casein. However, SMC reaches maximum levels about 2 days later than β -casein, suggesting that its expression has a different regulatory mechanism. Interestingly, the SMC transcript is present at similar levels in virgin and pregnant mammary gland, in contrast to the expression of β -casein transcript, which only becomes detectable around day 6 of pregnancy (6). The expression pattern of SMC *in vivo*, where the level of protein does not directly correspond to the level of transcript, suggests a post-transcriptional mechanism of regulation for this protein.

To study the mechanism of SMC regulation and the factors which may influence SMC expression in normal mammary gland, we utilized primary mammary epithelial cell culture. Primary culture of mammary epithelial cells has been used to elucidate the regulatory mechanisms of several milk proteins. These systems are thought to mimic the *in vivo* state and are useful because the level of differentiation/functionality can be maintained or manipulated. For example, in the presence of Matrigel and lactogenic hormones (insulin, hydrocortisone, and prolactin), isolated mammary epithelial cells will form round alveolar structures with closed lumens and vectorially secrete milk proteins (4, 28, 32–34). On plastic these cells form squamous epithelial monolayers and are considered undifferentiated because milk proteins are not synthesized (5).

Expression of β -casein in isolated mammary epithelial cells is dependent on the presence of extracellular matrix compo-

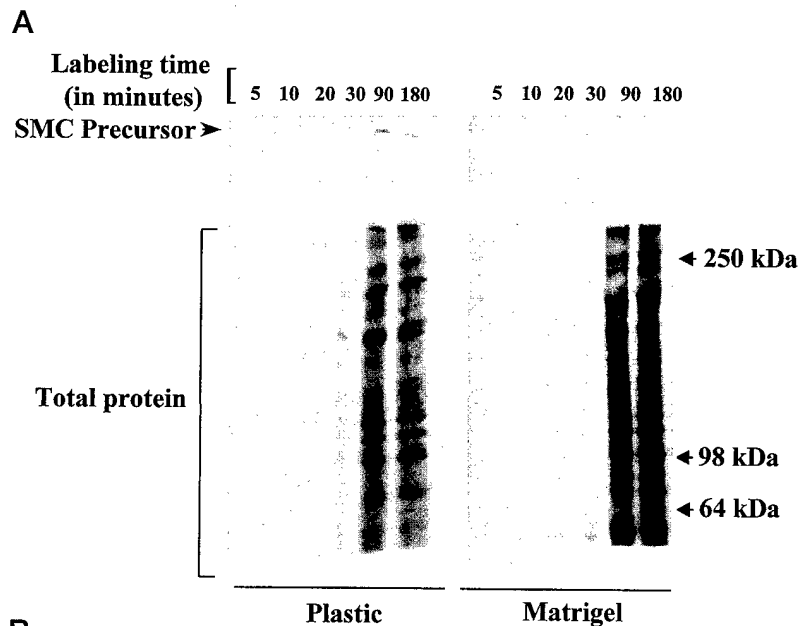


FIG. 7. Effect of Matrigel on SMC precursor synthesis in normal rat mammary epithelial cells. Rat mammary epithelial cells were collected by collagenase digestion of virgin rat mammary tissue. Epithelial cells were plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence of 10% FCS. After 24 h, cells were labeled for the times indicated with [^{35} S]Met + [^{35} S]Cys. Cells were lysed in 2% SDS, diluted into a Triton X-100 buffer, and immunoprecipitated with anti-ASGP-2 polyclonal antibody and protein A-agarose. Immunoprecipitates were subjected to SDS-PAGE and fluorography. **A**, fluorogram of immunoprecipitated SMC precursor and total protein. **B**, difference in SMC translation rate in the presence or absence of Matrigel. The SMC precursor band and its corresponding total protein lane from the 90-min labeling sample were quantified by densitometry. The ratios of SMC precursor to total protein were plotted in **B**.

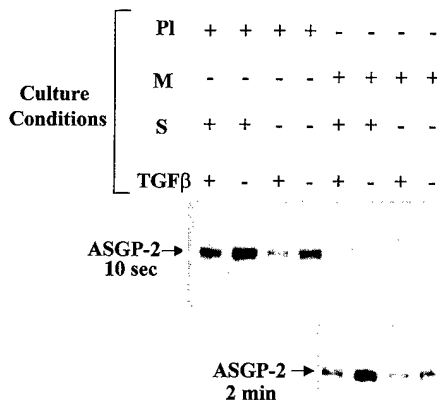
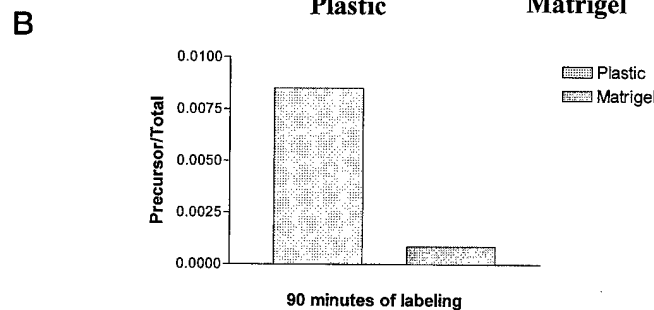


FIG. 8. Effect of TGF β on SMC protein levels in normal MEC. Normal rat MEC were isolated and cultured either on plastic or embedded in Matrigel in the presence or absence of 10% FCS and 5 ng/ml TGF β as indicated at the top of the figure. Cell were lysed and the lysates were subjected to immunoblot analysis with anti-ASGP-2 mAb 4F12. Note: exposure time is indicated at the left of the figure. Samples from cells cultured in Matrigel were exposed for 2 min as compared with 10 s for cells cultured on plastic in order to detect a significant amount of staining. Pl, plastic; M, Matrigel; S, serum.

nents (or basement membrane). In the presence of a reconstituted basement membrane (Matrigel), mammary epithelial cells from pregnant mice and virgin rats can be induced to express β -casein (5, 29). There are several major differences in the behavior of β -casein and SMC in primary MEC cultures in the presence or absence of basement membrane. 1) SMC is found at the highest levels when pregnant rat MEC are cultured on plastic and at very low levels when the cells are embedded in Matrigel. β -Casein is found at maximal levels

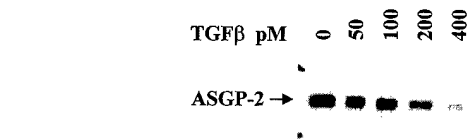


FIG. 9. Dose response of SMC to TGF β in normal rat MEC. Normal rat MEC were isolated and cultured on plastic under serum-free conditions with varying concentrations of TGF β as indicated at the top of the figure. Cell lysates were subjected to immunoblot analysis with anti-ASGP-2 mAb 4F12.

when pregnant rat MEC are embedded in Matrigel and only at minimal levels when the cells are cultured on plastic. 2) SMC transcript levels are not affected by MEC culture on plastic or in Matrigel, while β -casein transcript levels have been shown to be low (or non-existent) when MEC are cultured on plastic and high when MEC are embedded in Matrigel (35). 3) SMC protein production can be induced in MEC from virgin rats within hours of culture on plastic, suggesting that MEC do not need to be primed by pregnancy to be capable of synthesizing SMC. β -Casein expression, on the other hand, requires MEC priming by pregnancy or long-term culture under specific conditions for expression (29, 30). Primary culture of normal rat MEC does seem to mimic the *in vivo* state with respect to SMC expression (as well as β -casein expression, as shown by others). Furthermore, these data suggest that SMC is regulated by a post-transcriptional mechanism.

There are also several differences in SMC expression in normal MEC and the 13762 rat mammary adenocarcinoma, in which SMC was first identified. The 13762 rat mammary adenocarcinoma ascites cells express SMC at a level at least 100-fold higher than that in normal lactating mammary gland (8).

1) Unlike normal MEC, which express SMC at high levels upon removal from the animal, the MAT-B1 tumor cells significantly reduce SMC expression within 48 h during culture after re-

moval from the animal. 2) Culture in Matrigel does not seem to affect SMC levels in MAT-B1 tumor cells, either at the level of protein or transcript. In normal MEC, however, Matrigel reduces SMC levels in newly cultured normal MEC and in MEC that already express SMC. It appears that regulation of SMC expression in MAT-B1 tumor cells has been disrupted compared with that of normal MEC. In the MAT-B1 cells the transcript and protein are both synthesized equally well on plastic or in Matrigel, while in normal MEC the transcript is made but protein synthesis is reduced significantly in Matrigel. We have no evidence to suggest that SMC from the tumor cells is different from SMC from normal cells. The maintenance of SMC in the presence of Matrigel in the tumor confirms that the reduction of SMC seen in normal MEC is not due to the presence of a protease in the Matrigel. More likely, the tumor cells have lost their responsiveness to Matrigel.

Post-transcriptional regulation of milk proteins has not received substantial attention. TGF β can decrease casein production in mammary organ explants without affecting its transcript level (32). SMC synthesis in normal rat MEC is also suppressed by TGF β with no effect on its transcript level. This result suggests that TGF β may be responsible for regulating both casein and SMC post-transcriptionally in the normal developing mammary gland. Some other post-transcriptional regulation mechanisms have also been described. For example, it has been reported that β -casein transcript is stabilized by the presence of prolactin (36). WAP, a late milk protein, requires MEC culture in a three-dimensional matrix and a hollow alveolar structure with a closed lumen for expression (6). However,

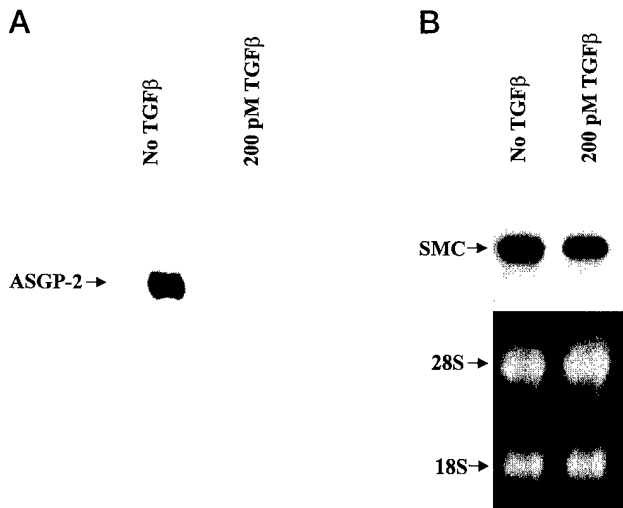
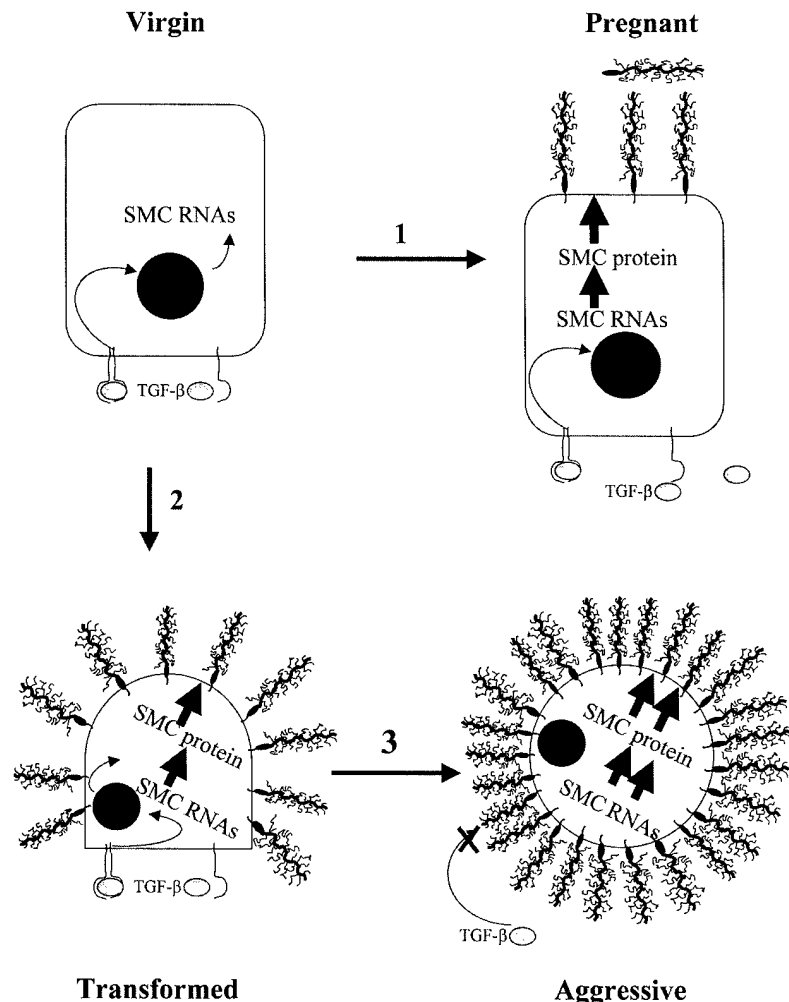


FIG. 10. Effect of TGF β on SMC transcript levels in normal MEC. Normal rat MEC were isolated and cultured on plastic in serum-free medium in the presence or absence of 10 ng/ml TGF β . Total RNA was isolated and 25 μ g of total RNA subjected to Northern blot analysis with probe A2G2-9. **A**, immunoblot with mAb 4F12 indicates a large decrease in the level of SMC when MEC are cultured in the presence of TGF β . **B**, Northern blot with probe A2G2-9 indicates no significant change in SMC message when MEC are cultured in the presence of TGF β .

FIG. 11. Model for regulation of SMC expression in normal mammary epithelial cells and its disruption in mammary tumor cells. In normal virgin MEC SMC RNA is present at high levels, but there is some modification or inhibitor regulated by TGF β that blocks translation of the SMC message. Thus, SMC protein is present at very low levels in virgin MEC. 1, during pregnancy, as the ECM, hormone, and growth factor milieu changes, the TGF β effect is overridden or TGF β is down-regulated, allowing for biosynthesis of SMC protein. 2, at some point in neoplastic transformation, TGF β responsiveness is lost, SMC begins to be overexpressed, and the cell loses polarity. 3, as the tumor becomes more aggressive and SMC is grossly overexpressed, cell-cell and cell-matrix interactions are disrupted and there is loss of recognition by the immune system, allowing for metastasis of the aggressive tumor. The mechanism by which the TGF β effect is overcome during pregnancy and disrupted in the 13762 ascites tumor cells is currently under investigation.



there is no difference between WAP transcription on plastic or on basement membrane, suggesting another post-transcriptional regulatory mechanism (6).

SMC, which is apparently the rat homolog of human MUC4, is expressed in other tissues besides mammary gland. SMC has a similar tissue distribution to that reported for MUC4. SMC is constitutively expressed in normal rat trachea, colon goblet cells, and small intestinal Paneth cells (8, 17, 37, 38). Human MUC4 gene expression has also been in numerous epithelial tissues, including trachea, small and large intestine, and cervix (23). SMC expression in the uterus appears to be under the control of the ovarian hormones estrogen and progesterone at the transcript level (22). Suppression of SMC synthesis by TGF β is probably not the only mechanism for regulating its expression in normal mammary gland. The complex tissue distribution and expression pattern of SMC in normal rat tissues suggests that it has multiple and complex regulatory mechanisms.

Regulation of milk proteins is achieved through complex interactions of hormones, growth factors, and extracellular matrix components (1–5). Many studies have been done to sort out the regulatory pathways for β -casein and WAP, and from these, many specific factors have been shown to play roles in complex cascades of events leading to functional differentiation of mammary tissue. For example, β -casein expression depends largely on the lactogenic hormone prolactin and the extracellular matrix component laminin (7, 39). β -Casein expression does not require cell-cell contact or closed alveolar structures, and its expression is inhibited by the growth factors TGF β and EGF (4, 5, 40–42). WAP, on the other hand, requires cell-cell contact in the formation of closed alveolar structures in an ECM, the glucocorticoid hydrocortisone, and the down-regulation of the growth factor TGF α (4, 39, 43). SMC is at least partially regulated by TGF β in normal mammary gland. Studies are currently underway to determine other factors or combinations of factors which may regulate SMC levels in normal mammary tissue.

The data presented here suggest that SMC, a product of differentiated mammary tissue, appears to be regulated post-transcriptionally in normal mammary tissue. There are several ways protein production can be regulated post-transcriptionally. These include stability of the transcript, changes in the rate of message translation, and changes in the rate of protein degradation or turnover. The steady state levels of SMC transcript were unaffected either by developmental state *in vivo* or by culture conditions *in vitro*, suggesting that SMC transcript stability in these conditions is unchanged. Because the level of SMC protein is significantly reduced in Matrigel, it appears that SMC protein is turning over in the cultured cells. Moreover, when cells cultured on plastic or Matrigel are metabolically labeled and SMC precursor is immunoprecipitated and quantified relative to total labeled protein, SMC is translated at ~8-fold higher rate on plastic than in Matrigel. Changes in SMC protein stability under these culture conditions are being investigated. However, taken together these results suggest that SMC biosynthesis is post-transcriptionally regulated in normal MEC by a factor present in Matrigel, most likely TGF β .

Other explanations could be proposed for differences in SMC transcript and protein expression in the virgin mammary gland and for the apparent decrease of SMC levels in the presence of Matrigel and TGF β . One possibility is that the monoclonal antibody 4F12 (which recognizes an epitope in the N-terminal 53 amino acids of ASGP-2) used to detect SMC may not recognize alternative splice forms of ASGP-2 or forms that have post-translational modification, such as differential phosphorylation or glycosylation. However, other monoclonal antibodies

against ASGP-2 that recognize other epitopes in the central portion of ASGP-2 (more C-terminal) yield similar results to those presented (data not shown), suggesting that these are unlikely possibilities. In addition, previous attempts to demonstrate alternative splice forms of SMC in normal rat tissues or the ascites tumor cells have been unsuccessful (8). Another possibility is that there is proteolytic cleavage of SMC producing secreted forms of SMC, which are not detected. We have reported detection of secreted SMC from lactating mammary tissue by serial immunoprecipitation with polyclonal antibodies against the C-terminal peptide followed by immunoprecipitation with polyclonal antibodies against total SMC to precipitate any SMC not recognized by the C-terminal antibody (8). Furthermore, we can also detect similar ratios of the membrane bound and secreted forms of SMC in the cultured virgin mammary epithelial cells used in these studies (data not shown). Therefore, although these possibilities cannot be entirely ruled out, our data strongly suggest that the antibodies used in the studies reported here do recognize all forms of SMC and that the apparent decrease in the level of SMC in the presence of Matrigel and TGF β is due to a real decrease in SMC protein levels under these conditions.

From these studies we can propose the following model. Virgin rat mammary epithelial cells are primed for SMC production by the continual presence of SMC transcript, but the transcript is not translated because of some RNA modification or translational inhibitor under the control of the TGF β pathway (Fig. 11). As pregnancy proceeds and the growth factor, hormone, and ECM milieu change, this modification or inhibition is altered, the suppression of SMC synthesis by TGF β is released and SMC synthesis can occur. Upon removal of MEC from the animal the repression is also relieved, presumably by removing TGF β from the epithelial cell environment, allowing biosynthesis of SMC under the culture conditions we have described.

In the MAT-B1 tumor cells this level of regulation appears to have been disrupted, and this, in combination with the 5-fold amplification of the gene and overexpression of the transcript contributes to its gross overexpression in this tumor cell line (15) (Fig. 11). TGF β may be involved in keeping the expression of this protein in check in the normal animal. Loss of TGF β responsiveness is a significant factor in tumor progression, and may be involved in the overexpression of SMC in the MAT-B1 ascites tumor cells. Loss of TGF β suppression thus may contribute to SMC overexpression in these cells, along with other factors. The growth factor-like properties of SMC may then contribute to uncontrolled proliferation of these cells, while the mucin subunit contributes to protection from the immune system and loss of adhesion. In summary, SMC is an unusual post-transcriptionally regulated milk membrane protein, whose overexpression contributes properties conducive to tumor progression. Elucidation of its regulatory mechanism by TGF β and other factors in normal developing mammary gland and its disruption in the 13762 tumor cells will give further insight into both normal developmental processes and tumor progression. Since translational regulation might be involved, investigation of factors regulating translation (44) should be of interest, including the 0.3-kilobase 3'-untranslated region of the transcript.

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