Prolactin Negatively Regulates Caveolin-1 Gene Expression in the Mammary Gland during Lactation, via a Ras-dependent Mechanism*

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Caveolin-1 is a 22-kDa integral membrane protein that has been suggested to function as a negative regulator of mitogen-stimulated proliferation in a variety of cell types, including mammary epithelial cells. Because much of our insight into caveolin-1 function has come from the study of human breast tumor-derived cell lines in culture, the normal physiological regulators of caveolin-1 expression in the mammary gland remain unknown. Here, we examine caveolin-1 expression in mice at different stages of mammary gland development. We show that caveolin-1 expression is significantly down-regulated during late pregnancy and lactation. Upon weaning, mammary gland expression of caveolin-1 rapidly returns to non-pregnant “steady-state” levels. Injection of virgin mice with a battery of hormones normally up-regulated during lactation demonstrates that prolactin is the major mediator of caveolin-1 down-regulation. Virtually identical results were obtained with human mammary epithelial cells (hTERT-HME1) in culture. In addition, we demonstrate that prolactin-mediated down-regulation of caveolin-1 expression occurs at the level of transcriptional control and via a Ras-dependent mechanism. Interestingly, in the mammary gland, both mammary epithelial cells and the surrounding mammary adipocytes show prolactin-mediated down-regulation of caveolin-1. This hormone-dependent regulation of caveolin-1 expression is specific to the mammary fat pad. Finally, we employed HC11 cells, a well-established model of mammary epithelial cell differentiation, to study the possible functional effects of caveolin-1 expression. In the presence of lactogenic hormones, recombination expression of caveolin-1 in HC11 cells dramatically suppresses the induction of the promoter activity and the synthesis of β-casein, an established reporter of lactogenic differentiation and milk production. These findings may explain why caveolin-1 levels are normally down-regulated during lactation. This report is the first demonstration that caveolin-1 levels are down-regulated during a normal physiological event in vivo, i.e. lactation, because previous reports have only documented that down-regulation of caveolin-1 occurs during cell transformation and tumorigenesis.

The mammary gland is one of the few organs that undergoes numerous rounds of proliferation and regression throughout adult life. Normal mammary gland development involves a complex interplay among growth factors, steroids, proto-oncogenes, and tumor suppressor genes (1–3). Development of the adult mammary gland can be divided into four distinct stages: non-pregnant, gestation, lactation, and involution. Gestation is characterized by rapid lobulo-alveolar outgrowth, whereas further proliferation and functional differentiation of the secretory epithelium are hallmarks of lactation. Finally, the end of weaning suppresses lactation and leads to involution of the lobulo-alveolar compartment, returning the mammary gland to its non-pregnant state (4). Dysregulation of these constituents can lead to mammary epithelial hyperplasia and ultimately to mammary tumorigenesis (5).

A possible role for caveolin-1 in mammary tumorigenesis was first identified using differential display and subtractive hybridization techniques. Sager and colleagues (6) identified a number of “candidate tumor suppressor genes”; these are genes whose mRNAs were down-regulated in human mammary adenocarcinoma-derived cells. In this screening approach, caveolin-1 was independently identified as one of 26 gene products down-regulated during mammary tumorigenesis. In addition, caveolin-1 expression was absent in several transformed cell lines derived from human mammary carcinomas, including MT-1, MCF-7, ZR-75-1, T47D, MDA-MB-361, and MDA-MB-474. In contrast, caveolin-1 mRNA is abundantly expressed in normal mammary epithelium (6, 7).

Since these initial observations, an increasing body of evidence has accumulated that supports the idea that caveolin-1 may function as a tumor suppressor in the mammary gland. For example, Lee et al. (8) demonstrated that exogenous expression of caveolin-1 in human breast cancer-derived cells (T47D) leads to a 50% reduction in cell proliferation and a 15-fold reduction in anchorage-independent growth in soft agar. Furthermore, p53-null fibroblasts express virtually undetectable levels of caveolin-1 (8).

Mutation and/or amplification of the NEU proto-oncogene occurs in a significant number of human mammary tumors (up to 30%). Mutational activation of the NEU proto-oncogene

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(NEU T) was shown to have a reciprocal relationship with caveolin-1 expression. Activated mutants of the c-Neu protein expressed in NIH-3T3 and Rat1 cells caused a dramatic down-regulation of caveolin-1 protein expression. In addition, mammary tumors derived from Neu transgenic mice displayed a dramatic reduction in caveolin-1 expression. Conversely, recombiant expression of caveolin-1 is sufficient to block Neumediated signal transduction, via the caveolin-1 scaffolding domain (5).

Caveolin-1 expression also diminishes the metastatic potential of the mammary tumor cell line, MTLn3. MTLn3 cells were originally derived from a metastatic rat mammary adenocarcinoma. In response to EGF,1 MTLn3 cells undergo lamellipodia extension and increased chemotaxis. However, recombinant expression of caveolin-1 in MTLn3 cells blocks EGF-induced lamellipodia extension and cell migration. Thus, expression of caveolin-1 in MTLn3 cells induces a non-motile phenotype (9).

To further investigate the possible clinical significance of caveolin-1 in mammary tumorigenesis, Hayashi et al. (10) screened 92 human breast cancer samples for a mutation in the caveolin-1 gene. Their results indicated a mutation at residue 132 (P132L) in 16% of the samples tested. The mutation most closely correlated with invasive scirrhous carcinomas. NIH-3T3 cells, stably expressing the caveolin-1 (P132L) mutant, demonstrated increased growth in soft agar, hyperactivation of the Ras-p42/44 MAPK cascade, and an altered cellular morphology due to disruption of the actin cytoskeleton. These results emphasize the importance of wild-type caveolin-1 gene expression in the normal regulation of mammary epithelial cell proliferation (10).

From the above studies, it is clear that caveolin-1 assumes a dynamic role in regulating mammary epithelial cell proliferation. However, the normal physiological regulators of caveolin-1 expression in vivo remain obscure. To identify these in vivo regulators, we analyzed the expression levels of caveolin-1 in the adult mouse mammary gland during different stages of its development. Through this analysis, we expected to find a stage of development in which caveolin-1 levels are normally up-regulated or down-regulated. This would allow us to then identify the hormonal regulators that control caveolin-1 expression.

Here, we demonstrate that caveolin-1 expression is significantly down-regulated in the mammary gland during late pregnancy and lactation. Injection of virgin mice with a battery of hormones normally up-regulated during lactation demonstrates that prolactin is the main mediator of caveolin-1 down-regulation. Furthermore, we show that prolactin negatively regulates caveolin-1 expression via a Ras-p42/44-MAPK-dependent mechanism. Finally, we employed HC11 cells, a well-established model of mammary epithelial cell differentiation, to study the possible functional effects of caveolin-1 expression. In the presence of lactogenic hormones, recombiant expression of caveolin-1 in HC11 cells dramatically suppresses the induction of both β-casein promoter activity and β-casein synthesis, as revealed by quantitative RT-PCR. β-Casein is an established marker of differentiation and milk production. This report is the first demonstration that caveolin-1 levels are down-regulated during a normal physiological event in vivo, because previous reports have only documented that down-regulation of caveolin-1 occurs during cell transformation and tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Caveolin-1 mouse mAb 2297 and caveolin-2 mouse mAb 65 (used for immunoblotting (11, 12)) were the gifts of Dr. Roberto Campos-Gonzalez, Transduction Laboratories, Inc. Anti-PPAR-γ antibodies were purchased from Santa Cruz Biotechnology; the anti-β-actin mAb (clone AC-15) was purchased from Sigma. hTERT-HME1 cells, a telomerase-immortalized, human mammary epithelial cell line, was purchased from CLONTECH (13, 14). HC11 cells, derived from the COMMA-D cell line, were the generous gift of Dr. J. M. Rosen, Baylor College of Medicine (Houston, TX). The permission of Dr. B. Groner, at The Friedrich Miescher Institute (Basel, Switzerland); COMMA-D cells were first isolated from the mammary glands of mice in mid-pregnancy. A variety of other reagents were purchased commercially. Cell culture reagents were from Life Technologies, Inc. Oxine prolactin (o-prolactin), dexamethasone, and insulin were purchased from Sigma Chemical Co. Recombinant human EGF was purchased from Upstate Biotechnology, Inc. The MEK inhibitor, PD98059, was purchased from Calbiochem.

**Cell Culture**—hTERT-HME1 cells were grown in complete growth medium consisting of MCDB-170 medium supplemented with 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 10 ng/ml human EGF, 5 μg/ml insulin, and 50 μg/ml gentamicin (Clontech). Cells were maintained in growth medium at 37 °C and 5% CO₂. Prior to hormone treatment, cells were grown to ~80% confluency, washed with PBS, and incubated in phenol red-free Dulbecco’s modified Eagle’s medium complete medium with 10% charcoal-dextran-stripped fetal bovine serum (PBF-CDS DMEM) for 12 h. Cells were then treated with increasing concentrations of oxytocin (0–1000 nM) or o-prolactin (0–100 μg/ml) for 72 h. To assess MEK involvement, PD98059 (50 μM) was added 24 h prior to o-prolactin treatment. HC11 cells were grown to confluency in RPMI 1640 medium supplemented with 10% donor calf serum, insulin (5 μg/ml), and epidermal growth factor (EGF, 10 ng/ml). For lactogenic hormone induction, the cells were maintained at confluence for 3 days in growth medium. HC11 cells were then primed in RPMI 1640 medium supplemented with 10% charcoal-dextran stripped horse serum and insulin (5 μg/ml) for 24 h. For hormonal induction, the following hormones were added to priming medium: dexamethasone (1 μg/ml) and o-prolactin (5 μg/ml) (15, 16).

**Hormone Treatment of Animals**—C57BL/6 mice (10–12 weeks old, Jackson Laboratories) were treated once a day with subcutaneous injections of 1 IU of oxytocin or 10 IU of prolactin for three consecutive days. All hormones were dissolved in 200 μl of PBS. On the fourth day, mice were given a final injection and sacrificed 2 h later (17). Mammary glands were excised and subjected to immunoblot and/or Northern blot analysis as detailed below.

**Expression Vectors**—The cDNA encoding caveolin-1 was subcloned into the multiple cloning site (HindIII/BamHI) of the cytomegalovirus-driven pCB7 vector, as described previously (18). The β-casein promoter-luciferase reporter was as characterized previously (19). Adenoviral vectors (Ad-Cav-1, Ad-GFP, and Ad-tTA) were as described previously (9).

**Immunoblot Analysis**—Cells were cultured in their respective media and allowed to reach ~80–90% confluency. Subsequently, they were washed with PBS and treated with lysis buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1% Triton X-100, 60 mM octyl glucoside) containing protease inhibitors (Roche Molecular Biochemicals). For protein isolation from tissue, 100 mg of the excised mammary gland was homogenized in lysis buffer, followed by immunoblot analysis. All subsequent wash buffers contained 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, which was supplemented with 1% bovine serum albumin and 1% nonfat dry milk powder for the blocking solution and 1% bovine serum albumin for the antibody diluent. Primary antibodies were used at a 1:50 dilution. Horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution, Pierce) were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (Pierce).

**Northern Blot Analysis**—Total RNA was extracted from 100 μg of tissue from each sample using the TRizol reagent protocol (Life Tech
nologies, Inc.). Twenty micrograms of total RNA for each sample was separated using a 1.2% agarose gel under RNase-free conditions and transferred to nitrocellulose. The filters were hybridized using the ExpressHyb solution (CLONTECH). The blots were probed with the radiolabeled caveolin-1 cDNA.

In Vivo Reporter Assay—A 13-kb DNA segment containing the caveolin-1 exons 1 and 2 was identified by screening a mouse genomic DNA library as previously described (20). A portion of caveolin-1 exon 1 in addition to intron 1 and the 3-kb upstream promoter were derived from this segment and subcloned into the vector p3SLUC, a promoter-less vector containing the luciferase cDNA as a reporter (21, 22). In this way, the effect of various signal transduction pathways on the regulation of caveolin-1 at the transcriptional level could be assessed. Transient transfections were performed using LipofectAMINE Plus (Life Technologies, Inc.). Briefly, 150,000 hTERT-HME1 or HC11 cells were seeded in 6-well plates 12–24 h prior to transfection. Each well was then transfected with 1.0 μg of the indicated luciferase reporter and 0.2 μg of pSV-β-gal (Promega). The pSV-β-gal, an SV40-driven vector expressing β-galactosidase, was used as a control for transfection efficiency. 0.5 μg of pCB7 or pCB7-caveolin-1 was co-transfected where indicated. For hTERT-HME1 cells: At 12 h post-transfection, the cells were rinsed once with PBS and incubated in PRF-CDS DMEM with 10 μg/ml prolactin for 48 h. The cells were lysed in 200 μl of extraction buffer, 100 μl of which was used to measure luciferase activity, as described previously (20). Another 50 μl of the lysate was used to conduct a β-galactosidase assay, as described previously (24). Each experimental value has been normalized using its respective β-galactosidase activity and represents the average of two separate transfections performed in parallel; the error bars in the figures represent the observed standard deviation. All experiments were performed at least three times independently and yielded virtually identical results. For HC11 cells: see above for hormone treatment.

Adenoviral Infection—Conditions for adenoviral transduction of cells were optimized by immunofluorescence and immunoblot analysis so that relatively high protein expression was achieved without toxicity to the cells. Twenty-four hours prior to infection, ~3 × 10^6 HC11 cells were plated in 10-cm dishes. At the time of infection, cells were washed once with PBS and incubated for 1 h with serum-free media containing either Ad-Cav-1 + Ad-TA (100 + 100 plaque-forming units/cell, respectively) or Ad-GFP + Ad-TA (100 + 100 plaque-forming units/cell, respectively). Cells were then washed with PBS and maintained in HC11 growth media.

RT-PCR—Total cellular RNA was extracted from HC11 cells using the TRizol reagent (Life Technologies, Inc., Rockville, MD). Total RNA (2 μg) was reverse-transcribed with Moloney murine leukemia virus-RT (Life Technologies, Inc., Life-Technologies, Inc.) oligo(dt) primers at 37 °C for 1 h. β-Casein-specific primers for RT-PCR were as described previously (1): CasYP (ACT ACA TTT ACT GTA TCC TCT GCC), nucleotides 107–130, and CasYM (GTG CTT TGG CCA GAA AGT ACA G), nucleotides 620–644. CDNAs were amplified under the following conditions: initially 2 min at 94 °C, 28 or 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension for 5 min at 72 °C (1). The glyceraldehyde-3-phosphate dehydrogenase cDNA was used as a positive internal control, with RGAPH (GTG AAG GTC GTG ACG GAA TGG CCG CTG), nucleotides 50–76, and RGAPH-2 (CCA CCC TCT TGC TGT AG), nucleotides 997–1016. As a negative internal control, equal amounts of RNA were amplified for 28 or 35 cycles using CasYP and CasYM without reverse transcription (1).

Separation of Mammary Epithelial Cells and Mammary Adipocytes—Mammary epithelial cells and adipocytes were separated as described elsewhere (25). Briefly, mammary glands (4 and 5) were excised from 10- to 12-week-old virgin, day 15 pregnant, day 10 lactating, and day 4 post-weaning C57Bl/6 mice. Samples were then immersed in Hanks’ balanced saline solution supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml). Tissue samples were minced and digested using type I collagenase (1 mg/ml). Digestions were performed in a 50-cc conical tube on a shaker at 37 °C for 30–60 min. After collagenase treatment, the slurry was passed through a 350-μm nylon mesh, and samples were centrifuged at 500 × g for 1 min to separate the parenchymal fraction from the floating adipocyte population. The floating samples were washed three times in Hanks’ balanced saline solution (25). The samples were then treated with lysis buffer (see above) and examined by immunoblot analysis.

RESULTS

Hormonal Regulation of Caveolin-1 Expression during Mammary Gland Development: Caveolin-1 Is Down-regulated during Late Pregnancy and Lactation—Because caveolin-1 has been shown to act as an inhibitor of mitogenesis in cultured cells, we examined the expression of caveolin-1 during mammmary gland development. Rapid expansion of the lobulo-alveolar compartment occurs during pregnancy and continues throughout lactation. Therefore, we initially examined caveolin-1 and caveolin-2 protein levels during these developmental stages of the mammary gland. C57Bl/6 mice between the ages of 10 and 12 weeks were sacrificed on day 16 of pregnancy and day 10 of lactation, with virgin mice being sacrificed as controls. The mammary glands were then excised, and caveolin protein levels were assessed by Western blotting. As shown in Fig. 1, initial screening revealed that caveolin-1 protein levels are significantly down-regulated during lactation, whereas caveolin-2 levels are not as dramatically affected.

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Fig. 1. Caveolin-1 protein expression is down-regulated in the mammary gland during lactation. C57Bl/6 mice between the ages of 10 and 12 weeks were sacrificed at different stages of mammary gland development. The mammary glands were then excised, whole cell lysates were prepared, and caveolin-1 and -2 protein levels were analyzed by Western blotting. Blotting with anti-β-actin IgG was performed as a control for equal protein loading. Each time point represents an average of three mice. Note that caveolin-1 protein levels are significantly down-regulated during lactation, whereas caveolin-2 levels are not as dramatically affected.

To determine at what molecular level lactation regulates...
caveolin-1 expression, Northern blot analyses were performed on the same mammary gland samples. As Fig. 3 illustrates, caveolin-1 mRNA transcript levels fall dramatically with the onset of lactation. On the third day of weaning, transcript levels begin to re-emerge and then fully return to non-pregnant levels by day 6 of weaning. To examine if the caveolin-1 protein is secreted into milk, samples were collected and examined for the presence of caveolin-1. However, no detectable levels of caveolin-1 were found in milk samples (not shown).

Prolactin Negatively Regulates Caveolin-1 Gene Expression in the Mammary Gland

During pregnancy, a complex array of ovarian and pituitary hormones, such as progesterone, prolactin, and oxytocin, stimulate the development of the lobulo-alveolar compartment within the mammary gland. Upon parturition, progesterone levels fall sharply, while prolactin and oxytocin levels remain elevated; this is a hallmark of the beginning of lactation. Suckling maintains high levels of prolactin and oxytocin secretion throughout lactation (26). To dissect the hormonal regulation of caveolin-1 expression observed in the lactating mammary gland, 10- to 12-week-old virgin mice were treated with subcutaneous injections of prolactin, oxytocin, or PBS alone. Fig. 4 demonstrates that, although oxytocin treatment moderately decreases caveolin-1 expression in comparison with PBS-treated mice, prolactin injection markedly reduced the caveolin-1 protein to nearly undetectable levels.

To further characterize the effects of prolactin and oxytocin on mammary epithelial cells, hTERT-HME1 cells were utilized. hTERT-HME1 cells are derived from primary human mammary epithelial cells that have been immortalized by stable transfection with human telomerase. This particular cell line has been well characterized and displays expression patterns and behaviors similar to non-immortalized primary mammary epithelial cells (13, 14). Consistent with the idea that hTERT-HME1 cells are immortalized but not oncogenically transformed, these cells abundantly express caveolin-1. There is a distinct lack of caveolin-1 expression in all previously studied breast cancer-derived cell lines. Therefore, this cell line is ideal for studying the effects of various hormones on caveolin-1 expression in culture.

bTERT-HME1 cells were treated with increasing concentrations of oxytocin (0–1000 nM) or α-prolactin (0–100 μg/ml) in
Note that down-regulation of caveolin-1 protein levels occurs with increasing prolactin concentrations, whereas oxytocin treatment shows no effect (Fig. 5). Furthermore, caveolin-2 protein levels are remarkably unaffected by prolactin or oxytocin treatment. These results directly show that prolactin is the main mediator of caveolin-1 down-regulation in mammary epithelial cells.

Caveolin-1 RNA transcript levels were shown to be down-regulated in mammary glands during lactation (Fig. 3). To determine whether this repression is due to increased RNA instability or to a decrease in transcription, an in vitro reporter assay was performed using a 3-kb portion of the caveolin-1 promoter sub-cloned into a luciferase reporter construct (18). This caveolin-1 promoter construct was used to transiently transfect hTERT-HME1 cells. Transfected cells were left untreated or treated with 10 μg/ml prolactin. As shown in Fig. 6, treatment with prolactin decreases caveolin-1 promoter activity by greater than 2-fold. Therefore, prolactin negatively regulates caveolin-1 expression, at least in part, via transcriptional control.

**Down-regulation of Caveolin-1 Expression Occurs via Prolactin-mediated Activation of the Ras-p42/44 MAPK Cascade—**

The prolactin receptor is a single-pass transmembrane receptor that belongs to the class I cytokine receptor super-family (2, 27). Although the receptor has no intrinsic kinase activity, activation of the prolactin receptor leads to tyrosine phosphorylation of several signaling molecules. Ligand binding leads to activation of Janus kinase 2 (JAK-2), which transphosphorylates other JAK-2 molecules and phosphorylates the prolactin receptor. The tyrosine-phosphorylated prolactin receptor then serves as a scaffolding protein for the recruitment of SH2-containing molecules, such as signal transducers and activators of transcription (STAT) proteins (27).

In addition to the JAK/STAT pathway, the liganded prolactin receptor has been shown to activate the Ras-p42/44 MAPK pathway through the adapter proteins, Shc/Grb2/SOS (27). To investigate which of these signaling pathways is involved in the down-regulation of caveolin-1 gene expression, the caveolin-1 promoter was screened for STAT- and/or MAPK-responsive binding elements. Utilizing the TESS search algorithm, an analysis of the mouse and human caveolin-1 promoter sequences 3 kb upstream revealed a distinct lack of STAT consensus elements (20). On the other hand, an Elk-1-binding site is located ~450 bp upstream from the ATG start site. In addi-
Caveolin-1 Down-regulation Occurs in Both Mammary Epithelial Cells and Mammary Adipocytes, but Not in Peri-uterine Adipocytes—The adult mammary gland is composed of two major components: the fatty stroma (adipocytes) and the parenchyma (epithelial cells). The virgin mammary gland is characterized by a predominance of stromal fat; however, during gestation and lactation, the lobulo-alveolar compartment rapidly expands and the gland fills with epithelial cells. Whether the down-regulation of caveolin-1 expression is due to the change in the ratio of mammary adipocytes to epithelial cells was examined by physically separating the stromal and parenchymal compartments.

This was achieved by collagenase treatment of mammary glands from virgin, pregnant, lactating, and weaned mice. The samples were then centrifuged to separate the floating adipocyte fraction from the pelleted epithelial cell fraction. As an internal control, peri-uterine fat pads were also excised from the same mice. As shown in Fig. 8A, caveolin-1 protein levels were down-regulated during lactation in isolated mammary epithelial cells. To verify the lack of contaminating stromal adipocytes in the epithelial fraction, the samples were also immunoblotted for an adipocyte-specific marker, PPAR-γ. Note the distinct lack of PPAR-γ expression in the epithelial cell samples.

Interestingly, caveolin-1 expression in the isolated mammary adipocytes is also repressed during lactation (Fig. 8A). The degree of caveolin-1 suppression appears to be similar to that seen in the epithelial cell fraction. To confirm that equal amounts of adipocyte protein were loaded, PPAR-γ expression was used as a control for equal protein loading.

Furthermore, as shown in Fig. 8B, caveolin-1 expression in the peri-uterine fat pad is unaffected during lactation. This reinforces the concept that different fat pads are functionally distinct. As demonstrated here, mammary adipocytes and peri-uterine adipocytes are clearly under the control of different hormonal stimuli.

Caveolin-1 Inhibits β-Casein Synthesis in HC11 Mammary Epithelial Cells upon Differentiation—The ability of caveolin-1 to inhibit β-casein synthesis was further examined using HC11 cells, which display the essential features of functionally differentiated mammary epithelial cells. In these studies, we used an adenoviral vector to efficiently deliver the caveolin-1 cDNA to HC11 cells. HC11 cells were transiently transfected with the β-casein promoter-luciferase construct and either the caveolin-1 cDNA or empty vector. As shown in Fig. 9, the cells were then treated with either dexamethasone and insulin or dexamethasone, insulin, and o-prolactin. Note that, in the absence of caveolin-1, β-casein promoter activity increased 3-4-fold in the presence of o-prolactin. However, when caveolin-1 is recombinantly expressed, β-casein promoter activity does not increase in response to prolactin treatment. Also note that caveolin-1 expression even lowers baseline β-casein promoter activity, indicating that caveolin-1 is a potent negative regulator of prolactin-induced phenotypic changes.

Caveolin-1 Inhibits β-Casein Synthesis in HC11 Mammary Epithelial Cells upon Differentiation—The ability of caveolin-1 to inhibit β-casein synthesis was further examined using HC11 cells, which display the essential features of functionally differentiated mammary epithelial cells. In these studies, we used an adenoviral vector to efficiently deliver the caveolin-1 cDNA (Ad-Cav-1). In addition, this adenoviral vector system is inducible and requires a co-activator for expression (Ad-tTA, as previously described (9)). Another adenovirus, harboring GFP, was used as a control to rule out the nonspecific effects of protein overexpression (Ad-GFP).

Briefly, HC11 cells were infected with Ad-Cav-1 plus Ad-tTA, Ad-GFP plus Ad-tTA, or left uninfected. The cells were then treated with a mixture of lactogenic hormones-with prolactin (+) or without prolactin (−) for 60 h. RT-PCR was then performed on the total RNA extracted from the infected HC11 cells using β-casein-specific primers or glyceraldehyde-3-phosphate dehydrogenase-specific primers, as a positive internal control.
As shown in Fig. 10, only transduction with Ad-Cav-1 plus Ad-tTA inhibited β-casein expression. In contrast, the Ad-GFP plus Ad-tTA maintained β-casein expression equivalent to the uninfected cells. Thus, recombinant expression of the caveolin-1 protein is sufficient to inhibit β-casein synthesis, a marker for prolactin-induced mammary epithelial cell differentiation.
caveolin-1 expression is at the transcriptional level, as demonstrated by using luciferase reporter assays and Northern blot analyses. Prolactin negatively regulates caveolin-1 expression via a Ras-dependent mechanism, because the MEK inhibitor, PD98059, abrogates this regulation. Interestingly, prolactin-mediated down-regulation of caveolin-1 occurs both in mammary epithelial cells and the surrounding mammary adipocytes. However, this hormone-dependent regulation of caveolin-1 expression is specific to the mammary fat pad, because caveolin-1 expression in the peri-uterine fat pad remains unaffected under the same conditions. Furthermore, recombinant expression of caveolin-1 in HC11 cells potently suppresses \( \beta \)-casein promoter activity and \( \beta \)-casein synthesis, demonstrating for the first time that caveolin-1 can negatively modulate prolactin-mediated activation of the JAK/STAT signaling cascade.

Prolactin is synthesized predominantly in the anterior pituitary. It is a peptide hormone that belongs to group I of the helix-bundle protein hormones, which consists of prolactin, growth hormone, and placental lactogen. First discovered in bovine pituitary extract due to its ability to elicit lactation in rabbits and growth of the crop sac and production of crop milk in pigeons, the critical role of prolactin in mammmoapoeiosis and lactogenesis is now being fully realized (27). Gene deletion experiments have been carried out at multiple levels of the prolactin-signaling cascade, including prolactin, prolactin receptor, STAT5a, and STAT5b. Knock-out mice of each of these molecules, except STAT5b, demonstrate a severe impairment of mammary gland development and lactation (4, 29, 30).

The critical role that prolactin plays in mammary gland growth and development has obvious implications for mammary tumorigenesis. Early studies implicating pituitary hormones in the development of breast cancer came from patients with metastatic breast carcinoma who were surgically treated with trans-sphenoidal hypophysectomy. The study revealed that, of the 50 patients treated, 58% experienced objective response (31). Furthermore, Kwa et al. (32) and others (2) found an association with elevated prolactin levels in families with a high prevalence of breast cancer.

Although the results of other clinical trials have been less promising for the role that prolactin plays in breast cancer, these studies have mainly focused only on prolactin secreted from the pituitary gland (33, 34). However, recent studies have demonstrated that the autocrine and paracrine stimulation of locally produced prolactin may have more significance for breast cancer than the pituitary-derived prolactin (35, 36). It is now known that breast cancer cells and cell lines, as well as normal lactating mammary epithelial cells, are able to produce and secrete prolactin (27, 35, 36).

In light of these findings, several groups have demonstrated that breast cancers and breast tumor cell lines up-regulate prolactin receptor expression —2- to 15-fold over non-transformed controls (37–39). To test the functional significance of prolactin receptor expression in these cells, Fur and Wells (37) treated various breast cancer cell lines with prolactin receptor antagonists, which bind to the receptor but do not allow dimerization. Importantly, antagonist treatment led to growth inhibition in the breast cancer cell lines tested (37).

Most convincingly, however, was the demonstration that prolactin transgenic mice (but not mice expressing bovine growth hormone) developed spontaneous mammary adenocarcinomas after ~12 months of age. In addition, mammary organ cultures from prolactin transgenic mice revealed that locally produced prolactin was able to maintain lobulo-alveolar outgrowth in mammary explants. Furthermore, when wild-type mammary explants were co-cultured with prolactin transgenic mammary explants, wild-type mammary explants demonstrated lobulo-alveolar proliferation (3).

The finding that caveolin-1 expression is under the negative regulation of prolactin in the mammary gland in vivo provides novel insights into the signaling pathways that caveolin-1 may be regulating endogenously. As mentioned previously, the prolactin receptor is a cytokine receptor that primarily acts through the JAK2/STAT5 signaling cascade but also activates the Ras-p42/44 MAPK cascade and phosphatidylinositol 3-kinase (PI3K) pathways (27). It is possible that caveolin-1 may regulate prolactin receptor signaling cascades at multiple levels in mammary gland development.

The reciprocal relationship between the Ras-p42/44 MAPK pathway and caveolin-1 has been well established. Treatment with mitogens, such as PDGF, or expression of constitutively activated H-Ras(G12V) in NIH-3T3 cells negatively regulates caveolin-1 expression at the transcriptional level (5, 18, 20, 28, 40). On the other hand, caveolin-1 expression antagonizes the effects of activated Ras by acting on its downstream effectors, such as Raf, MEK, and ERK. Caveolin-1 has been shown to negatively regulate both Ras-p42/44-MAPK-mediated and basal transcriptional activation of mitogen-sensitive promoters. Also the caveolin-1-scaffolding domain, residues 82–101, was shown to directly inhibit the kinase activity of MEK-1 and ERK-2 in vitro (18). Abolishing caveolin-1 expression in NIH-3T3, using an antisense approach, led to increased anchorage-independent growth in soft agar and tumor formation in immunodeficient mice, as well as hyperactivation of the Ras-p42/44 MAPK cascade (28).

The re-emergence of caveolin-1 expression during involution, a period of rapid resorption of the lobulo-alveolar compartment, may lend an additional mechanism to the accelerated rate of apoptosis seen in lobulo-alveolar epithelial cells. Zundel and colleagues (41) have shown that caveolin-1 plays a critical role in down-regulating the PI3K/Akt survival pathway. Overexpression of caveolin-1 altered PI3K activity and increased ceramide-stimulated apoptosis in fibroblasts. On the other hand, antisense caveolin-1 dramatically reduced ceramide-dependent PI3K regulation and closely resembled acid-sphingomyelinase-deficient cells, which are resistant to stress-induced apoptosis (41). In addition, our group has demonstrated that NIH-3T3 cells harboring antisense caveolin-1 are resistant to staurosporine-induced apoptosis as determined by cell morphology, DNA content, caspase-3 activation, and focal adhesion kinase cleavage (42). Furthermore, T24 bladder carcinoma cells stably expressing caveolin-1 are sensitized toward apoptotic stimuli (42).

The ability of exogenous caveolin-1 expression to abrogate \( \beta \)-casein promoter activity and \( \beta \)-casein synthesis in response to prolactin represents a novel function for caveolin-1, i.e. as a negative regulator of the JAK2/STAT5 signaling pathway. Although caveolin-1 also negatively regulates the Ras-p42/44 MAPK pathway, Wartmann et al. (15) have thoroughly demonstrated that phosphorylation of STAT5 and transcriptional activation of the \( \beta \)-casein gene are independent of the p42/44 MAPK pathway. These findings are consistent with caveolin-1 expression patterns seen in the developing mammary gland. Caveolin-1 protein levels become significantly down-regulated near parturition and are almost absent throughout lactation, possibly to allow prolactin to activate the JAK2/STAT5 cascade. Furthermore, involution is marked by a brisk resurgence of caveolin-1 protein levels, which is consistent with the loss of STAT5 phosphorylation and cessation of lactation (43).

In summary, we show that caveolin-1 protein expression is transcriptionally down-regulated in mammary epithelial cells during lactation. Prolactin mediates this repression in vivo and
in vitro via a Ras-dependent mechanism. Furthermore, exogenous caveolin-1 expression is able to suppress the induction of prolactin-stimulated β-casein promoter activity and β-casein synthesis in cultured mammary epithelial cells.

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