Caveolin-1-deficient Mice Show Accelerated Mammary Gland Development During Pregnancy, Premature Lactation, and Hyperactivation of the Jak-2/STAT5a Signaling Cascade

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It is well established that mammary gland development and lactation are tightly controlled by prolactin signaling. Binding of prolactin to its cognate receptor (Prl-R) leads to activation of the Jak-2 tyrosine kinase and the recruitment/tyrosine phosphorylation of STAT5a. However, the mechanisms for attenuating the Prl-R/Jak-2/STAT5a signaling cascade are just now being elucidated. Here, we present evidence that caveolin-1 functions as a novel suppressor of cytokine signaling in the mammary gland, akin to the SOCS family of proteins. Specifically, we show that caveolin-1 expression blocks prolactin-induced activation of a STAT5a-responsive luciferase reporter in mammary epithelial cells. Furthermore, caveolin-1 expression inhibited prolactin-induced STAT5a tyrosine phosphorylation and DNA binding activity, suggesting that caveolin-1 may negatively regulate the Jak-2 tyrosine kinase. Because the caveolin-scaffolding domain bears a striking resemblance to the SOCS pseudosubstrate domain, we examined whether Jak-2 associates with caveolin-1. In accordance with this homology, we demonstrate that Jak-2 cofractionates and coimmunoprecipitates with caveolin-1. We next tested the *in vivo* relevance of these findings using female Cav-1 (–/–) null mice. If caveolin-1 normally functions as a suppressor of cytokine signaling in the mammary gland, then Cav-1 null mice should show premature development of the lobuloalveolar compartment because of hyperactivation of the prolactin signaling cascade via disinhibition of Jak-2. In accordance with this prediction, Cav-1 null mice show accelerated development of the lobuloalveolar compartment, premature milk production, and hyperphosphorylation of STAT5a (pY694) at its Jak-2 phosphorylation site. In addition, the Ras-p42/44 MAPK cascade is hyper-activated. Because a similar premature lactation phenotype is observed in SOCS1 (–/–) null mice, we conclude that caveolin-1 is a novel suppressor of cytokine signaling.

INTRODUCTION

Development of the adult mammary gland has been divided into four distinct stages: virgin, pregnancy, lactation, and

involution. During pregnancy, the mammary gland undergoes rapid lobuloalveolar outgrowth, whereas further proliferation and functional differentiation of the secretory epithelium are hallmarks of lactation. Weaning of the young initiates involution of the lobuloalveolar compartment, returning the mammary gland to its nonpregnant state (Hennighausen and Robinson, 1998). The tight regulation of this

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developmental process requires a complex interplay of steroid and peptide hormones.

Prolactin functions as a key modulator of mammary epithelial growth and differentiation during pregnancy and lactation. It is a peptide hormone synthesized in the anterior pituitary and belongs to group I of the helix-bundle protein hormones, which includes prolactin, growth hormone, and placental lactogen (Freeman *et al.*, 2000). Binding of prolactin to its cognate receptor (Prl-R) leads to recruitment and activation of Janus kinase 2 (Jak-2), leading to phosphorylation of the Prl-R. The phosphorylated receptor then acts as a scaffolding protein for activating signaling complexes, such as Ras/mitogen-activated protein kinase (MAPK) and signal transducers and activators of transcription (STAT5) (Hennighausen and Robinson, 1998; Freeman *et al.*, 2000). Gene deletion experiments have been carried out at multiple levels of the prolactin-signaling cascade and lead to a severe impairment of mammapoiesis and lactation (Liu *et al.*, 1997; Hennighausen and Robinson, 1998; Goffin *et al.*, 1999).

Although prolactin signaling in the mammary gland has been well characterized, the mechanisms for attenuating this cascade are just beginning to be elucidated. One such mechanism is via the suppressors of cytokine signaling (SOCS1). SOCS1 inhibits Jak-2/STAT5a signaling by directly competing with endogenous substrates for the Jak-2 kinase domain (Lindeman *et al.*, 2001). We now present evidence that caveolin-1 serves as a negative regulator of the Jak-2/STAT5a pathway both in vitro and in vivo.

The mammalian caveolin gene family consists of caveolins 1, 2, and 3 (Parton, 1996; Scherer *et al.*, 1996; Tang *et al.*, 1996; Okamoto *et al.*, 1998). Caveolins 1 and 2 are coexpressed and form a hetero-oligomeric complex (Scherer *et al.*, 1997) in many cell types, with particularly high expression in adipocytes, endothelial cells, fibroblasts, and epithelial cells (Rothberg *et al.*, 1992; Scherer *et al.*, 1996), whereas the expression of caveolin-3 is muscle-specific (Tang *et al.*, 1996). Cav-1 and Cav-3 are both independently necessary and sufficient to drive caveola formation in heterologous expression systems, whereas Cav-2 requires the presence of Cav-1 for proper membrane targeting and stabilization. In the absence of Cav-1, Cav-2 localizes to the Golgi complex, where it is degraded by the proteasomal system (Parolini *et al.*, 1999; Razani *et al.*, 2001). It has been proposed that caveolin family members function as scaffolding proteins (Sargiacomo *et al.*, 1995) to organize and concentrate specific lipids (cholesterol and glycosphingolipids) (Fra *et al.*, 1995; Murata *et al.*, 1995; Li *et al.*, 1996c) and lipid-modified signaling molecules (Src-like kinases, H-Ras, eNOS and G-proteins) (Garcia-Cardena *et al.*, 1996; Li *et al.*, 1996a,b,c; Shaul *et al.*, 1996; Song *et al.*, 1996) within caveola membranes.

Each caveolin-interacting protein binds to the same membrane-proximal cytoplasmic region of Cav-1, called the caveolin-scaffolding domain (CSD, residues 82–101) (Li *et al.*, 1996a; Couet *et al.*, 1997). Cav-1 interacts with heterotrimeric G-protein α subunits, H-Ras, Src-family tyrosine kinases, epidermal growth factor receptor (EGF-R), Neu, protein kinase (PK) C isoforms, PKA, and endothelial nitric oxide synthase (eNOS) via this scaffolding domain (for review, see Okamoto *et al.*, 1998). Binding of these signaling molecules to the CSD inhibits their enzymatic activity, and mutations that constitutively activate signaling proteins abolish interactions with the CSD.

We previously demonstrated that Cav-1 expression is dramatically down-regulated during late pregnancy and lactation (Park *et al.*, 2001). This Cav-1 downregulation event is mediated by the Prl-R signaling cascade, but via a Ras-p42/44 MAPK-dependent mechanism that inhibits Cav-1 gene transcription (Park *et al.*, 2001). Because Cav-1 has been suggested to function as a negative regulator of mitogen-stimulated proliferation in a variety of cell types, including mammary epithelial cells, we have begun to assess the ability of Cav-1 to suppress prolactin receptor signaling. Interestingly, our preliminary results demonstrated that recombinant overexpression of Cav-1 in HC11 cells was sufficient to inhibit prolactin-induced activation of β -casein promoter activity and synthesis (Park *et al.*, 2001). However, the mechanism by which Cav-1 exerts this inhibitory activity remains unknown.

Here, we demonstrate that Cav-1 blocks β -casein promoter activity and synthesis by functioning as a negative regulator of the Jak-2/STAT5a signaling pathway. We show that recombinant expression of Cav-1 in HC11 cells represses prolactin-induced activation of a Stat5a-responsive promoter construct. To assess whether Cav-1 interacts with members of the Prl-R signaling pathway in vivo, caveolin-rich membrane domains were purified from whole mammary gland. Jak-2 was found to cofractionate with caveolae and to coimmunoprecipitate with Cav-1 in the mammary gland. In accordance with these observations, we show that the primary sequence of the Cav-1 scaffolding domain is strikingly similar to the SOCS pseudosubstrate domain, exhibiting a series of highly conserved residues. Consistent with this homology, heterologous expression of Cav-1 in HC11 cells inhibited prolactin-induced STAT5a phosphorylation and DNA binding activity.

We also explored the in vivo relevance of these findings using female Cav-1 null (–/–) mice. If caveolin-1 normally functions as a suppressor of cytokine signaling in the mammary gland, we would predict that Cav-1 null mice should show premature development of the lobuloalveolar compartment because of hyperactivation of the prolactin signaling cascade via disinhibition of Jak-2. In direct support of this hypothesis, whole-mount analysis of Cav-1 null mammary glands revealed accelerated development of the lobuloalveolar compartment during pregnancy, with precocious lactation. Biochemical analyses of mouse mammary glands demonstrated that in Cav-1 null mice, the expression of milk proteins (α -casein, β -casein, and whey acidic protein [WAP]) was premature by ~2–3 d as compared with their wild-type counterparts. To determine whether changes in prolactin signaling are responsible for accelerated lobuloalveolar development and lactation in Cav-1 null mice, we next examined the activation state of key signaling molecules that are located downstream of the prolactin receptor, using phospho-specific antibody probes. Interestingly, we show that STAT5a is prematurely activated and hyperphosphorylated in Cav-1-deficient mammary glands; similarly, p42/44 MAPK is hyperactivated. Cav-1 deficiency also led to sustained activation of STAT5a during involution. Taken together, these data provide in vivo support for the hypothesis that caveolin-1 normally functions as a negative regulator of the Prl-R/Jak-2/STAT5a signaling cascade in the mammary gland.

MATERIALS AND METHODS

Materials

Caveolin-1 mouse monoclonal antibody (mAb) 2297 (used for immunoblotting) (Scherer *et al.*, 1995, 1997) was the generous gift of Dr. Roberto Campos-Gonzalez, BD-Transduction Laboratories. Antibodies that specifically recognize total STAT5a and activated STAT5a (phospho-STAT5a, pY694) were purchased from BD-Transduction Laboratories. Antibodies directed against total extracellular signal-regulated kinase (ERK)-1/2 and activated phospho-ERK-1/2 were obtained from Cell Signaling (a subsidiary of NEB). Anti-Jak-2 was purchased from Upstate Biotechnology, Lake Placid, NY. The anti-prolactin receptor antibody was from Affinity Bioreagents, Inc. A rabbit polyclonal antiserum raised against mouse milk-specific proteins (α -casein, β -casein, and WAP) was purchased from Accurate Chemical and Scientific Corp. HC11 cells, derived from the COMMA-D cell line, were the generous gift of Dr. J.M. Rosen, Baylor College of Medicine, Houston, TX, with 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 midpregnancy. Other reagents were obtained from the following commercial sources: cell culture reagents (Life Technologies, Gaithersburg, MD); ovine prolactin (o-prolactin), dexamethasone, and insulin (Sigma, St. Louis, MO); and recombinant human EGF (Upstate Biotechnology, Inc.).

Animal Studies

All animals were housed and maintained in a pathogen-free environment/barrier facility at the Institute for Animal Studies at the Albert Einstein College of Medicine under National Institutes of Health (NIH) guidelines. CAV-1 deficient mice were generated as we previously described (Razani *et al.*, 2001). CAV-1 $-/-$ mice were back-crossed into the C57Bl/6 strain from Jackson Laboratories for at least five generations. Wild-type and knockout mice were generated through heterozygous matings.

Cell Culture

HC11 cells were grown to confluence in RPMI 1640 medium supplemented with 10% donor calf serum, insulin (5 μ g/ml), and EGF (10 ng/ml). Before treatment with lactogenic hormones, the cells were maintained at confluence for 3 d in growth medium. HC11 cells were then primed in RPMI 1640 medium supplemented with 10% charcoal-dextran-stripped horse serum and insulin (5 μ g/ml) for 24 h. During hormone treatment, the following hormones were added to the priming medium: dexamethasone (1 μ g/ml) and o-prolactin (5 μ g/ml) (Wartmann *et al.*, 1996; Ali, 1998). hTERT-HME1 cells were grown in complete growth medium consisting of MCDB 170 medium supplemented with 52 μ g/ml bovine pituitary extract, 0.5 μ g/ml hydrocortisone, 10 ng/ml hEGF, 5 μ g/ml insulin, and 50 μ g/ml gentamicin (Clonetics). hTERT-HME1 cells were maintained in growth medium at 37°C and 5% CO₂. Before hormone treatment, hTERT-HME1 cells were grown to ~80% confluence, washed with PBS, and incubated in phenol red-free DME complete medium with 10% charcoal-dextran-stripped FBS (PRF-CDS DMEM) for 12 h. hTERT-HME1 cells were then treated with increasing concentrations of estrogen alone (0 to 10⁻⁸ M), progesterone alone (0 to 10⁻⁷ M), or both for 24 h.

Purification of Caveolar Membrane Fractions

Caveola-enriched membrane fractions were purified as we previously described (Lisanti *et al.*, 1994; Razani *et al.*, 2001). Approximately 400 mg of mammary tissue from virgin C57Bl/6 mice was placed in 2 ml of MBS (25 mM Mes, pH 6.5, 150 mM NaCl) containing 1% Triton X-100 and solubilized by using quick 10-s bursts of a rotor homogenizer and passing 10 times through a loose-fitting Dounce homogenizer. The sample was mixed with an equal volume

of 80% sucrose (prepared in MBS lacking Triton X-100), transferred to a 12-ml ultracentrifuge tube, and overlaid with a discontinuous sucrose gradient (4 ml of 30% sucrose, 4 ml of 5% sucrose, both prepared in MBS lacking detergent). The samples were subjected to centrifugation at 200,000 $\times$ g (39,000 rpm in a Sorval rotor TH-641) for 16 h. A light-scattering band was observed at the 5/30% sucrose interface. Twelve 1-ml fractions were collected, and 50- μ l aliquots of each fraction were subjected to SDS-PAGE and immunoblotting.

Expression Vectors

The cDNA encoding caveolin-1 was subcloned into the multiple cloning site (*Hind*III/*Bam*HI) of the CMV-driven pCB7 mammalian expression vector, as described previously (Scherer *et al.*, 1995; Engelman *et al.*, 1998a,b). The β -casein promoter-luciferase reporter was as characterized previously (Matsumura *et al.*, 1999). The 3 $\times$ D1-SIE1-Luc plasmid was constructed by subcloning three consecutive STAT5a-responsive elements from the cyclin D1 promoter into the plasmid, PSP72-luciferase, as described by Matsumura *et al.* (1999). Adenoviral vectors (Ad-cav-1, Ad-GFP, and Ad-tTA) were as we described previously (Zhang *et al.*, 2000).

Immunoblot Analysis

Cells were cultured in their respective media and allowed to reach ~80–90% confluence. 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 (Boehringer Mannheim). For protein isolation from tissue, 100 mg of mammary gland was homogenized in lysis buffer. Cell and tissue lysates were then centrifuged at 12,000 $\times$ g for 10 min to remove insoluble debris. Protein concentrations were quantified using the BCA reagent (Pierce), and the volume required for 10 μ g of protein was determined. Samples were then separated by SDS-PAGE (12.5% acrylamide) and transferred to nitrocellulose. The nitrocellulose membranes were stained with Ponceau S (to visualize protein bands), followed by immunoblot analysis. All subsequent wash buffers contained 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20, which was supplemented with 1% bovine serum albumin (BSA) and 2% nonfat dry milk (Carnation) for the blocking solution and 1% BSA for the antibody diluent. Primary antibodies were used at a 1:500 dilution. Horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution, Pierce) were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (Pierce).

In Vivo Reporter Assays

Transient transfections were performed using Lipofectamine Plus (Life Technologies). Briefly, HC11 cells were seeded in 6-well plates 12–24 h before transfection. Each well was then transfected with 1.0 μ g of the indicated luciferase reporter and 0.2 μ g of pSV- β -gal (Promega). The pSV- β -gal plasmid, an SV40-driven vector expressing β -galactosidase, was used as a control for transfection efficiency. Where indicated, 0.5 μ g of pCB7 or pCB7-caveolin-1 was cotransfected. The cells were then treated with lactogenic hormones for 24 h or left untreated. The cells were lysed in 200 μ l of extraction buffer, 100 μ l of which was used to measure luciferase activity, as described (Pestell *et al.*, 1994). Another 50 μ l of the lysate was used to conduct a β -galactosidase assay, as previously described (Subramaniam *et al.*, 1990). Each experimental value was normalized using its respective β -galactosidase activity and represents the average of two separate transfections performed in parallel; error bars represent the observed SD. All experiments were performed at least three times independently and yielded virtually identical results.

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 (our unpublished observations). Twenty-four hours before infection, $\sim 3 \times 10^6$ HC11 cells were plated in 10-cm dishes. At the time of infection, cells were washed once with PBS and incubated for 1 hour with serum-free medium containing either Ad-cav-1 + Ad-tTA (100 + 100 pfu/cell, respectively) or Ad-GFP + Ad-tTA (100 + 100 pfu/cell, respectively). Cells were then washed with PBS and maintained in HC11 growth medium.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed as described (Wartmann *et al.*, 1996), with minor modifications. Competent HC11 cells were infected with Ad-Cav-1 plus Ad-tTA or Ad-GFP plus Ad-tTA or were left uninfected and then treated with lactogenic hormones for 30 min or left untreated. Whole-cell extracts were then prepared in extraction buffer as described by Wartmann *et al.* (1996). Extracts were isolated from $\sim 10^8$ cells, divided into aliquots, and frozen immediately. Concentrations were determined using the BCA Protein Assay Reagent (Pierce Chemical). For a STAT5-specific band shift, 6 μ g of whole-cell extract was incubated with the Stat5 consensus sequence generated from the bovine β -casein promoter (5-AGATTCTAGGAATTCAATCC-3) (Wartmann *et al.*, 1996) (50,000 cpm, 5 fmol) for 30 min on ice in 20 μ l of EMSA buffer: 10 mM HEPES, pH 7.6, 2 mM NaHPO₄, 0.25 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl₂, 80 mM KCl, 2% glycerol, and 50 μ g/ml poly(dI-dC). STAT5-specific binding was assessed on a 4% polyacrylamide gel, prerun for 2 h at 200 V, in 0.25 $\times$ TBE (22.5 mM Tris borate, pH 8.0, 0.5 mM EDTA). The samples were electrophoresed for 1 h at 200 V, and the gels were vacuum-dried and exposed to film at -80°C for 12 h.

Coimmunoprecipitation of Caveolin-1 with Jak-2

Immunoprecipitation of endogenous Jak-2 was performed as follows. Approximately 100 mg of mammary gland tissue from virgin C57Bl/6 mice was solubilized in lysis buffer (see Immunoblotting), clarified by centrifugation at $15,000 \times g$ for 15 min, and precleared by incubation with protein A-Sepharose (Amersham Pharmacia) for 1 h at 4°C . Supernatants were then transferred to separate 1.5-ml microcentrifuge tubes containing anti-Jak-2 IgG (rabbit polyclonal antibody [pAb]) prebound to protein-A Sepharose; appropriate negative controls were included and consisted of beads alone or pre-immune serum prebound to protein-A Sepharose. After incubation rotating overnight at 4°C , the immunoprecipitates were washed three times with lysis buffer and subjected to immunoblot analysis with anti-caveolin-1 IgG (cl 2297; mouse mAb).

Whole-Mount Preparations

Fourth mammary glands (inguinal) were excised, spread onto glass slides, and fixed in Carnoy's fixative (6 parts 100% EtOH, 3 parts CHCl₃, 1 part glacial acetic acid) for 2–4 h at room temperature. The samples were then washed in 70% EtOH for 15 min and changed gradually to distilled water. Once hydrated, the mammary squashes were stained overnight in carmine alum (1 g carmine [Sigma C1022] and 2.5 g aluminum potassium sulfate [Sigma A7167] in 500 ml distilled water). The samples were then dehydrated using stepwise ethanol concentrations and defatted in xylenes. Mammary squashes were stored in methyl salicylate.

Radioimmunoassay of the Plasma Levels of PRL

Mouse serum samples were prepared from mice at indicated stages of pregnancy. Serum prolactin levels (ng/ml) were then determined using radioimmunoassay (Mills *et al.*, 2001), as prepared by the National Hormone and Peptide Program of the National Institute of Diabetes and Digestive and Kidney Diseases, directed by Dr. A. F. Parlow (Parlow@humc.edu).

RESULTS

Cav-1 Expression Negatively Regulates Prolactin-induced Activation of a STAT5a-specific Luciferase Reporter

Using mammary epithelial cells in culture, we have previously shown that expression of Cav-1 represses prolactin-induced β -casein transcription, a marker of lactogenic differentiation (Park *et al.*, 2001). Because growth and functional differentiation of the mammary epithelium is dependent primarily on the prolactin signaling cascade, these findings suggest that Cav-1 may function as a negative regulator of the prolactin receptor/Jak-2/STAT5a signaling pathway. However, this hypothesis remains untested.

Association of prolactin with its cognate receptor leads to receptor dimerization, recruitment of Jak-2, and the activation of STAT5a. Activation of STAT5a ultimately directs the synthesis of milk proteins, including β -casein. Therefore, we next assessed the ability of Cav-1 to specifically inhibit STAT5a activation by using a STAT5a-sensitive luciferase reporter construct after transient transfection of HC11 cells. This luciferase reporter, called 3 $\times$ D1-SIE1-Luc, contains a STAT5a-specific binding element (repeated 3 times) derived from the cyclin D1 promoter (Matsumura *et al.*, 1999).

HC11 cells, originally derived from the mouse mammary epithelial cell line COMMA-D, have become an established model system for studying mammary epithelial cell differentiation in culture. In the presence of lactogenic hormones (dexamethasone, insulin, and prolactin), HC11 cells assume a differentiated phenotype and express β -casein, an important milk protein and a marker for mammary epithelial cell differentiation (Wartmann *et al.*, 1996).

HC11 cells were transiently transfected with the 3 $\times$ D1-SIE1 luciferase reporter and either the Cav-1 cDNA (pCB7-Cav-1) or the vector alone control plasmid (pCB7). The cells were then treated with either dexamethasone and insulin (D/I), or dexamethasone, insulin, and o-prolactin (D/I/P). Figure 1A shows that in the absence of Cav-1 expression, 3 $\times$ D1-SIE1 luciferase activity rises approximately fourfold in response to o-prolactin treatment. However, when Cav-1 is expressed recombinantly, 3 $\times$ D1-SIE1 luciferase activity is no longer responsive to prolactin treatment. In fact, Cav-1 expression even lowers baseline 3 $\times$ D1-SIE1 luciferase activity, indicating that Cav-1 is a potent negative regulator of Jak-2/STAT5a signaling. The responses of a β -casein promoter-luciferase reporter are also shown for comparison (Figure 1A, left).

Jak-2 Cofractionates with Caveolar Membrane Domains and Coimmunoprecipitates with Cav-1

To examine the level at which Cav-1 might intersect with the Prl-R signaling cascade, caveolar membrane fractions were purified and probed for the presence of prolactin receptor, Jak-2, and STAT5a. Caveolin family members localize to specialized membrane microdomains known as "lipid rafts." Lipid rafts are enriched in cholesterol and sphingolipids and are resistant to detergent solubilization at low temperatures (Galbiati *et al.*, 2001). On the basis of their low-density and detergent resistance, caveola/lipid raft-enriched domains were purified from mammary glands of virgin C57Bl/6 mice using sucrose gradient fractionation, as

Figure 1. Cav-1 represses Jak-2/STAT5a signaling and associates with Jak-2. (A) STAT5a-responsive promoter activity. HC11 cells were transiently transfected with 1.0 μ g of 3 $\times$ D1-SIE1 Luc, 0.2 μ g of pSV- β -gal, and either pCB7-caveolin-1 or pCB7 (vector alone). The cells were then treated with a hormonal cocktail containing dexamethasone and insulin (DI) or dexamethasone, insulin, and o-prolactin (DIP). Note that in the absence of caveolin-1 expression, 3 $\times$ D1-SIE1-Luc activity rises approximately fourfold in response to o-prolactin treatment. However, when caveolin-1 is expressed re-

described in MATERIALS AND METHODS (Lisanti *et al.*, 1995). The resulting fractions were then subjected to immunoblot analysis to visualize the distribution of prolactin receptor, Jak-2, STAT5a, and Cav-1. Interestingly, as shown in Figure 1B, only Jak-2 cofractionates with Cav-1, whereas prolactin receptor and STAT5a are excluded from these caveolar fractions.

Because Jak-2 targets to caveolar/lipid raft-enriched membrane fractions, we next assessed the ability of Cav-1 to coimmunoprecipitate with Jak-2. Whole mammary tissue from virgin C57Bl/6 mice was homogenized in lysis buffer and incubated with protein A-Sepharose alone or in the presence of a Jak-2-specific pAb or a nonspecific pAb control. The samples were then subjected to immunoblot analysis with anti-Cav-1 IgG (mAb 2297). As shown in Figure 1C, Cav-1 specifically coimmunoprecipitates with the Jak-2-specific antibody but not with beads alone or preimmune serum. Immunoblotting with the Jak-2-specific pAb was also performed to confirm that Jak-2 was present in the immunoprecipitates (Figure 1C).

Thus, Cav-1 may interact with Jak-2 either directly or indirectly. However, because such a tight association is maintained during coimmunoprecipitation, we favor the notion that it is a direct interaction. The striking homology between the caveolin-scaffolding domain and the SOCS pseudosubstrate domain would also be more consistent with a direct interaction (see below; Figure 2).

Cav-1 Expression Functionally Inhibits Prolactin-induced STAT5a Activation, as Assessed by Tyrosine Phosphorylation and DNA-binding Activity

SOCS proteins negatively regulate the cytokine signaling cascades at multiple levels. In particular SOCS1, and to a lesser degree SOCS3, have been demonstrated to inhibit Jak-2 by directly binding its tyrosine kinase domain. Although the SOCS1 SH2 domain is sufficient for binding Jak-2, 12 residues N-terminal to the SH2 domain are necessary for inhibiting Jak-2 kinase activity. These 12 residues, found in both SOCS 1 and 3, resemble the Jak-activation loop and therefore serve as a pseudosubstrate (Yasukawa *et al.*, 1999; Krebs and Hilton, 2000).

combinantly, 3 $\times$ D1-SIE1 Luc activity is no longer responsive to prolactin treatment (*). The responses of a β -casein promoter-luciferase reporter are also shown for comparison (left). (B) Jak-2 cofractionates with caveolar membranes. Mammary tissue from virgin C57Bl/6 mice was homogenized and overlaid with a discontinuous sucrose gradient. The samples were centrifuged, and the resulting fractions were subjected to immunoblot analysis. Note that Prl-R and STAT5a are localized primarily in the noncaveolar membrane fractions, whereas Jak-2 cofractionates with Cav-1. (C) Coimmunoprecipitation of Cav-1 with Jak-2. Mammary tissue from virgin C57Bl/6 mice was homogenized in lysis buffer, precleared, and incubated with anti-Jak-2 IgG prebound to protein-A Sepharose or appropriate negative controls (beads alone, preimmune serum). Immunoprecipitates were then subjected to immunoblot analysis with anti-caveolin-1 IgG (mAb 2297) and anti-Jak-2 IgG. Note that Cav-1 coimmunoprecipitates with the Jak-2-specific antibody, but not with beads alone or preimmune serum. All of the above experiments were performed three times independently and yielded similar results.

Figure 2. Similarities between caveolins and the SOCS proteins. The caveolin protein family was screened for similarities to the SOCS pseudosubstrate domain. The CSD was found to have a series of conserved residues shared with the SOCS PSD. The conserved domains were characterized by the consensus sequence Φ TFxxS/T(+)xxxY(+), where Φ is a hydrophobic or aromatic amino acid and + is a positively charged residue. Interestingly, the scaffolding domains of both Cav-1 and Cav-3 fit this consensus sequence, whereas Cav-2 does not. This is consistent with previous observations showing that the scaffolding domains of Cav-1 and Cav-3 inhibit a wide variety of tyrosine and serine/threonine protein kinases, whereas the Cav-2 scaffolding domain does not possess this kinase inhibitory activity (for review, see Okamoto *et al.*, 1998; Smart *et al.*, 1999; Razani *et al.*, 2000).

Because Cav-1 can inhibit prolactin-induced STAT5a activation of a luciferase reporter (Figure 1A) and is physically associated with Jak-2 (Figure 1, B and C), the primary sequences of the caveolin gene family (Cav-1, -2, and -3) were screened for similarities to the SOCS pseudosubstrate domain (PSD). As shown in Figure 2, the caveolin-scaffolding domain bears a striking resemblance to the SOCS PSD, exhibiting a series of highly conserved residues with the following consensus motif: Φ TFxxS/T(+)xxxY(+), where Φ is a hydrophobic/aromatic amino acid and + denotes positively charged residues. Interestingly, the Cav-1 scaffolding domain has previously been shown *in vitro* to act as an inhibitor of both tyrosine and serine/threonine kinases, including receptor tyrosine kinases (EGF-R, platelet-derived growth factor receptor, ErbB2/Neu), nonreceptor tyrosine kinases (c-Src, Fyn), PKA, MAPKs (MEK and ERK), and certain protein kinase C isoforms (for review, see Okamoto *et al.*, 1998; Smart *et al.*, 1999; Razani *et al.*, 2000).

On the basis of the primary sequence similarities between the caveolin-scaffolding domain and the SOCS-pseudosubstrate domain, we next assessed the ability of Cav-1 to inhibit Jak-2 kinase activity in mammary epithelial cells. For these studies, we used an adenoviral vector to efficiently deliver the Cav-1 cDNA (Ad-Cav-1). This adenoviral vector system is inducible and requires a coactivator for expression (Ad-tTA) as previously described (Zhang *et al.*, 2000). Another adenovirus, harboring GFP (Ad-GFP), was used as a negative control to rule out the possible nonspecific effects of protein overexpression.

HC11 cells were infected with Ad-Cav-1 plus Ad-tTA or Ad-GFP plus Ad-tTA or were left uninfected. The cells were then treated with lactogenic hormones for 0, 5, and 30 min. Relative levels of STAT5a-tyrosine phosphorylation were determined by immunoblotting with a phospho-specific antibody probe that selectively recognizes activated STAT5a at

its Jak-2 phosphorylation site (pY694); phospho-independent anti-STAT5a IgGs were used as a control for equal loading. As shown in Figure 3A, only transduction with Ad-Cav-1 plus Ad-tTA inhibited prolactin-induced STAT5a-phosphorylation. In contrast, the cells transduced with Ad-GFP plus Ad-tTA maintained STAT5a phosphorylation equivalent to that of uninfected control cells. Thus, recombinant expression of the Cav-1 protein is sufficient to inhibit prolactin-induced STAT5a-phosphorylation, which is mediated by activation of the Jak-2 tyrosine kinase.

To examine whether this inhibition of STAT5a phosphorylation is functionally translated into decreased STAT5a activation, DNA binding of STAT5a in the presence of Cav-1 was determined by use of an EMSA. HC11 cells were infected with Ad-Cav-1 plus Ad-tTA or Ad-GFP plus Ad-tTA or were left uninfected. The cells were then treated with lactogenic hormones for 30 min or left untreated. Whole-cell extracts were then prepared and coincubated with an end-labeled STAT5a DNA-binding element from the bovine β -casein promoter (Wartmann *et al.*, 1996). The samples were then separated by electrophoresis on a nondenaturing gel. Figure 3B demonstrates that the cells transduced with Ad-GFP plus Ad-tTA were responsive to prolactin, which induced STAT5a DNA binding similar to that observed in uninfected control cells. However, transduction with Ad-Cav-1 plus Ad-tTA functionally inhibited the ability of prolactin to induce STAT5a DNA binding, as predicted.

Analysis of Cav-1 Null Mammary Glands Reveals Accelerated Development of the Lobuloalveolar Compartment during Pregnancy, with Precocious Lactation

The ability of Cav-1 expression to inhibit prolactin-induced STAT5a activation, as assessed by several independent approaches, prompted us to examine female Cav-1 null (–/–) mice for possible alterations in mammary gland development during pregnancy and lactation. If caveolin-1 normally functions as a suppressor of cytokine signaling in the mammary gland, we would predict that Cav-1 null mice should show premature development of the lobuloalveolar compartment because of hyperactivation of the prolactin signaling cascade via disinhibition of Jak-2.

Inguinal mammary glands from 8-wk-old Cav-1 +/+ and Cav-1 –/– female mice were examined at various time points during pregnancy using whole-mount analysis. Figure 4A demonstrates that Cav-1 –/– females exhibit accelerated lobuloalveolar development, as early as day 14 of pregnancy, compared with their wild-type counterparts. At day 18 of pregnancy, Cav-1 –/– mammary glands display dilated alveoli, characteristic of milk production, whereas wild-type mammary glands do not reach this level of alveolar development until lactation day 1.

To rule out the possibility that hyperactivation of the prolactin signaling cascade was simply a result of elevated circulating prolactin levels, serum samples were collected from wild-type and knockout mice during pregnancy and quantified by radioimmunoassay. Importantly, no statistical difference was noted in the serum prolactin levels of Cav-1 null mice as compared with their wild-type counterparts (Figure 4B).

Figure 3. Cav-1 expression inhibits prolactin-induced STAT5a activation, as measured by STAT5a tyrosine phosphorylation and STAT5a DNA binding activity. (A and B) HC11 cells were infected with Ad-Cav-1 plus Ad-tTA or Ad-GFP plus Ad-tTA or were left uninfected. (A) Phospho-STAT5a immunoblot analysis. After infection with the appropriate adenoviral vectors, cells were treated with lactogenic hormones for 0, 5, and 30 min. Whole-cell lysates were then prepared, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Relative levels of STAT5a-tyrosine phosphorylation were determined by immunoblotting with a phospho-specific antibody probe that selectively recognizes activated STAT5a at its Jak-2 phosphorylation site (pY694); phospho-independent anti-STAT5a IgGs were used as a control for equal loading. Note that only transduction with Ad-Cav-1 plus Ad-tTA inhibited prolactin-induced STAT5a phosphorylation (*). In contrast, the cells transduced with Ad-GFP plus Ad-tTA maintained STAT5a phosphorylation equivalent to that in uninfected cells. Thus, recombinant expression of the Cav-1 protein is sufficient to inhibit prolactin-induced STAT5a tyrosine phosphorylation. (B) EMSA. After infection with the appropriate adenoviral vectors, cells were treated with lactogenic hormones for 30 min or left untreated. Nuclear extracts were then prepared and coincubated with an end-labeled STAT5a DNA-binding element from the β -casein promoter. The samples were separated by electrophoresis on a nondenaturing gel. Note that cells transduced with Ad-GFP plus Ad-tTA were responsive to prolactin and showed prolactin-induced STAT5a DNA binding similar to that in uninfected control cells. However, transduction with Ad-Cav-1 plus Ad-tTA inhibited the ability of prolactin to induce STAT5a DNA binding (*). All of the above experiments were performed three times independently and yielded similar results.

As mentioned earlier, Cav-1 deficiency in mice leads to an ~95% reduction in Cav-2 protein levels, because Cav-1 protein expression is required to stabilize the Cav-2 protein product (Razani *et al.*, 2001). Therefore, Cav-1 null mice are essentially deficient in both Cav-1 and Cav-2. To determine whether the precocious lactation phenotype seen in Cav-1 null mice is a result of the loss of Cav-1 or Cav-2, we also examined Cav-2 null mice (Razani *et al.*, 2002) at various stages during pregnancy. Figure 4C shows that Cav-2 null mice do not show accelerated lobuloalveolar development. These results indicate that loss of Cav-1, and not Cav-2, is responsible for the accelerated mammary gland development seen in Cav-1 null mice.

Accelerated Milk Protein Production in Cav-1 $-/-$ Mammary Glands during Pregnancy

To compare alveolar development and milk globule content in Cav-1 $+/+$ and Cav-1 $-/-$ mammary glands, fourth mammary glands were formalin-fixed, sectioned, and stained with hematoxylin-eosin (H&E). Note that at day 18 of pregnancy, Cav-1 $-/-$ mammary glands are engorged with milk, whereas Cav-1 $+/+$ mammary glands are just beginning milk production (Figure 5A). At lactation day 1, Cav-1 $-/-$ females demonstrate alveolar wall thinning and further dilation of the alveoli, characteristic of several days

Figure 4. Cav-1 null mammary glands display accelerated development of the lobuloalveolar compartment during pregnancy, with precocious lactation. (A) Whole-mount analysis of Cav-1 null mammary glands. Cav-1 null and wild-type mammary glands were placed in Carnoy's fixative and stained with carmine dye. Note that Cav-1 $-/-$ females begin to demonstrate accelerated mammary development as early as day 14 of pregnancy. In addition, at day 18 of pregnancy, Cav-1 null mammary glands displayed dilated alveoli, characteristic of milk production. (B) Serum prolactin levels in Cav-1 null mice. Serum samples were collected from wild-type and Cav-1 null mice during pregnancy and quantified by radioimmunoassay. Importantly, no statistical differences were noted in serum prolactin levels of Cav-1 null mice as compared with their wild-type counterparts. Each time-point represents the average for a cohort of mice ($n \geq 8$ for each genotype). (C) Whole-mount analysis of Cav-2 null mammary glands. Cav-2 null and wild-type mammary glands were placed in Carnoy's fixative and stained with carmine dye. Note that whereas Cav-1 $-/-$ mammary glands display accelerated lobuloalveolar outgrowth (A), Cav-2 $-/-$ mammary glands progress at the same developmental rate as the wild-type samples (C). (A and C) The whole mounts shown for each time point are representative of a cohort of mice ($n = 3$ for each genotype); the experiments were performed three times independently and yielded similar results.

Figure 5. Accelerated milk protein production in Cav-1 $-/-$ mammary glands during pregnancy. Fourth mammary glands were harvested from wild-type and Cav-1 null mice (12 wk old) at different stages of pregnancy (days 16 and 18) or 1 day after the mice gave birth (Lact 1). To compare lobuloalveolar development and milk globule content in Cav-1 $+/+$ and Cav-1 $-/-$ mammary glands, fourth mammary glands were formalin-fixed, sectioned, and stained with H&E. (A) Low-magnification images (10 $\times$ objective). Note that at day 18 of pregnancy, Cav-1 $-/-$ mammary glands are engorged with milk, whereas Cav-1 $+/+$ mammary glands are just beginning milk production. (B) High-magnification images (40 $\times$ objective). At lactation day 1, Cav-1 $-/-$ females demonstrate alveolar wall thinning and further dilation of the alveoli, characteristic of several days of lactation, whereas Cav-1 $+/+$ mammary glands exhibit alveolar dilation representative of the first day of lactation. (A and B) The H&E-stained paraffin sections shown for each time point are representative of a cohort of mice ($n = 3$ for each genotype); the experiments were performed three times independently and yielded similar results.

of lactation, whereas Cav-1 $+/+$ mammary glands exhibit alveolar dilation representative of the first day of lactation (Figure 5B).

To biochemically assess milk protein production, we next performed immunoblot analysis using antisera raised against mouse milk proteins. As demonstrated in Figure 6A, Cav-1 $-/-$ mammary glands express milk proteins earlier than their wild-type counterparts. Note that the expression of α -casein, β -casein, and WAP in Cav-1 null mammary glands consistently preceded wild-type by ~ 2 –3 d. These biochemical results directly verify the Cav-1 null premature lactation phenotype we observed morphologically by whole-mount analysis and by H&E staining of mammary tissue sections.

Cav-1 Null Mammary Glands Show Premature Activation/Hyperphosphorylation of STAT5a and p42/44 MAPK during Pregnancy

Accelerated development of the lobuloalveolar compartment and premature lactation could be caused by hyperactivation of the Prl-R/Jak-2/STAT5a signaling pathway. To test this hypothesis, we used immunoblot analysis with a phospho-specific antibody probe to examine the activation

state of STAT5a in Cav-1 null mammary gland samples. As mentioned earlier, this phospho-specific antibody probe selectively recognizes activated STAT5a at its Jak-2 phosphorylation site (pY694); phospho-independent anti-STAT5a IgGs were also used as a control for equal loading. Figure 6B shows that Cav-1 null mammary glands clearly exhibit premature hyperphosphorylation of STAT5a during pregnancy, verifying that early mammary gland development in Cav-1 null mice is caused by hyperactivation of the Jak-2/STAT5a signaling pathway.

Because the Ras-p42/44 MAPK pathway is also activated by prolactin receptor signaling, mammary gland samples were subjected to immunoblot analysis with phospho-specific antibodies that specifically recognize activated ERK-1/2; phospho-independent anti-ERK-1/2 IgGs were used as a control for equal loading. Figure 6C shows that ERK-1/2 is hyperactivated during pregnancy in Cav-1 null mammary glands compared with their wild-type counterparts. In addition, the downregulation of ERK-1/2 activation, which typically marks the onset of milk production, occurs earlier in Cav-1-deficient mice. These findings are consistent with our previous observations that Cav-1 may also function as a natural endogenous inhibitor of the p42/44 MAPK cascade (Engelman *et al.*, 1998a; Galbiati *et al.*, 1998).

Figure 6. Biochemical analysis of milk protein production and signal transduction in Cav-1 null mammary glands. (A–C) Fourth mammary glands were harvested from wild-type and Cav-1 null mice (12 wk old) at different stages of pregnancy (days 14, 16, and 18) or 1 day after the mice gave birth (Lact 1). (A) Immunoblot analysis of milk protein production. Lysates were prepared from mammary glands at the indicated time points and subjected to immunoblot analysis with a rabbit polyclonal antibody directed against mouse milk protein components (α -casein, β -casein, and WAP) (from Accurate Chemical) (Lindeman *et al.*, 2001). Blotting with anti- β -actin IgG was performed as a control for equal protein loading. Note that Cav-1 $-/-$ mammary glands express milk proteins $\sim$ 2–3 d earlier than their wild-type counterparts. Thus, Cav-1 $-/-$ mice biochemically exhibit precocious mammary development during pregnancy, leading to premature lactation. (B and C) Analysis of STAT5a and p42/44 MAPK (ERK-1/2) activation. Lysates were prepared and subjected to immunoblot analysis with antibodies directed against phospho-STAT5a (B) and phospho-ERK (C). Immunoblots with phospho-independent antibodies to ERK and STAT5a are shown as controls for equal protein loading. Note that Cav-1 null mice show premature activation of STAT5a (especially during pregnancy, days 16 and 18 [P16 and P18]). In addition, Cav-1 null mice show premature activation of ERK-1/2 (especially during pregnancy, days 14 and 16 [P14 and P16]). Also, downregulation of ERK-1/2 activity, typically observed with the onset of milk production, occurs earlier (pregnancy, day 18 [P18]) in Cav-1 null mice. (D) Analysis of STAT5a activation during involution. Fourth mammary glands were harvested from wild-type and Cav-1 null mice (12 wk old) at different stages: 1) 1 d after the mice gave birth (lactation, day 1 [L1]) and 2) different times after forced weaning (involution, days 1, 3, and 6 [I1, I3, and I6]). Lysates were prepared and subjected to immunoblot analysis with antibodies directed against phospho-STAT5a; an immunoblot with phospho-independent antibodies to STAT5a is shown as a control for equal loading. Note the prolongation of STAT5a hyperphosphorylation by $\sim$ 2–3 d in Cav-1-deficient mammary samples. (A–D) Each time point represents a pooled cohort of $n = 3$ mice for each genotype; also, the above experiments were performed three times independently and yielded similar results.

Multiple lines of experimental evidence now indicate that Cav-1 functions as an endogenous inhibitor of the Ras-p42/44 MAPK cascade (Engelman *et al.*, 1997, 1998a; Galbiati *et al.*, 1998; Zhang *et al.*, 2000; Fiucci *et al.*, 2002). Thus, a negative reciprocal relationship exists between Cav-1 and the p42/44 MAPK cascade, because 1) Cav-1 expression is down-regulated by sustained activation of the Ras-p42/44 MAPK cascade at the level of transcriptional control (i.e., Cav-1 promoter studies) (Engelman *et al.*, 1999; Park *et al.*, 2001) and 2) the caveolin-1 scaffolding domain (residues 82–101) directly interacts with both MEK and ERK and inhibits their kinase activity (Engelman *et al.*, 1998a). Similarly, antisense-mediated ablation of Cav-1 expression in NIH 3T3 cells causes sustained hyperactivation of the Ras-p42/44 MAPK cascade (Galbiati *et al.*, 1998). Finally, RNA interference-based ablation of Cav-1 in *Caenorhabditis elegans* leads to progression of

the meiotic cell cycle, a phenotype that mirrors that of Ras activation (Scheel *et al.*, 1999).

Cav-1 Null Mammary Glands Show Sustained Hyperphosphorylation of STAT5a during Involution

We have previously demonstrated that Cav-1 expression is dramatically down-regulated during lactation; however, upon weaning, Cav-1 expression rapidly returns to non-pregnant “steady-state” levels (Park *et al.*, 2001). Thus, we assessed whether reexpression of Cav-1 during involution plays a role in negatively regulating Jak-2 activity by forced weaning and examination of the mammary glands at different time points. As shown in Figure 6D, mammary glands from Cav-1-deficient mice exhibited prolonged STAT5a

phosphorylation after weaning. However, we did not observe an extended period of lactation after the onset of weaning (as defined by morphological criteria) (our unpublished results). Thus, Cav-1 may not be directly involved in regulating the involution of the lobuloalveolar compartment. Alternatively, another as yet unknown compensatory mechanism may be at work in Cav-1 KO mice.

Estrogen and Progesterone Synergistically Up-regulate Cav-1 Protein Levels in Normal Mammary Epithelial Cells

We have previously shown that prolactin-mediated down-regulation of Cav-1 protein expression in the mammary glands of wild-type mice does not occur until day 18 of pregnancy (Park *et al.*, 2001), even though prolactin levels are highly up-regulated by day 14 of pregnancy. Therefore, additional mechanisms must be operating to maintain high levels of Cav-1 expression during pregnancy, thereby counteracting the effects of prolactin.

During pregnancy, estrogen and progesterone levels increase sharply, stimulating lobuloalveolar development within the mammary gland. On parturition, the levels of both steroid hormones decrease sharply, whereas prolactin levels remain elevated; this change marks the beginning of lactation (Hennighausen and Robinson, 1998). To characterize the effects of estrogen and progesterone on Cav-1 expression in mammary epithelial cells, hTERT-HME1 cells were used.

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 nonimmortalized primary mammary epithelial cells (Clontech, 2000a,b). Consistent with the idea that hTERT-HME1 cells are immortalized but not oncogenically transformed, these cells express Cav-1 abundantly. There is a distinct lack of Cav-1 expression in all previously studied breast cancer-derived cell lines (Zhang *et al.*, 2000). Therefore, this cell line is ideal for studying the effects of various hormones on Cav-1 expression in culture.

hTERT-HME1 cells were treated with increasing concentrations of estrogen alone (0 to 10^{-8} M), progesterone alone (0 to 10^{-7} M), or both in PRF-CDS DMEM. Note that treatment with estrogen alone yielded no change in Cav-1 expression (Figure 7A), whereas treatment with progesterone alone actually led to a mild repression of Cav-1 protein levels (Figure 7B). Interestingly, the combination of estrogen and progesterone produced a dose-dependent increase in Cav-1 protein expression (Figure 7C), providing a possible mechanism by which Cav-1 expression is maintained throughout pregnancy, despite high levels of prolactin.

DISCUSSION

The prolactin receptor is a single-pass transmembrane receptor that belongs to the class I cytokine receptor superfamily. First cloned in 1988, the identification of the cognate receptor of prolactin allowed for the identification of the intermediate molecules linking the receptor to target genes. The intermediate signaling cascades activated by the Prl-R

Figure 7. Estrogen and progesterone act synergistically to up-regulate Cav-1 protein expression in hTERT-immortalized human mammary epithelial cells (hTERT-HME1). hTERT-HME1 cells were preincubated in PRF-CDS DMEM for 12 h before treatment with either estrogen alone, progesterone alone, or both in graded concentrations. Note that treatment with estrogen alone yielded no change in Cav-1 expression (A), whereas treatment with progesterone alone (B) actually induced a mild repression of Cav-1 protein levels. Interestingly, a combination of estrogen and progesterone produced a dose-dependent increase in Cav-1 protein expression (C). Blotting with anti-β-actin IgG was performed as a control for equal protein loading. All of the above experiments were performed three times independently and yielded similar results.

Cav-1 and Prl-R/Jak-2/STAT5a Signaling

Figure 8. Caveolin-1 and Prl-R/Jak-2/STAT5a signaling. We have shown previously that prolactin induces the transcriptional downregulation of Cav-1 expression during late pregnancy, just prior to lactation. This prolactin-mediated downregulation of Cav-1 occurs via a Ras-p42/44 MAPK-dependent mechanism and is prevented by treatment of mammary epithelial cells with the MEK1/2 inhibitor PD 98059 (Park *et al.*, 2001). Conversely, we show here that Cav-1 normally functions as a negative regulator of Prl-R/Jak-2/STAT5a signaling, because 1) Cav-1 expression inhibits prolactin-induced STAT5a activation in cultured mammary epithelial cells (Figures 1–3) and 2) deletion of the Cav-1 gene in mice leads to hyperactivation of STAT5a and premature lactation (Figures 4–7). Importantly, this premature lactation phenotype appears to be Cav-1-specific, because Cav-2 null mice do not show premature development of the lobuloalveolar compartment (Figure 4C).

include the Jak-2/STAT5a, Ras-p42/44 MAPK, and the PI-3-kinase pathways (Freeman *et al.*, 2000). Despite our ever-increasing understanding of the signaling pathways governing mammary development and lactogenesis, there is a relative dearth of information on the processes that attenuate Prl-R signaling responses.

In this report, we provide *in vitro* and *in vivo* data implicating Cav-1 as a negative regulator of Jak-2/STAT5a signaling in the mammary gland. Mechanistically, we show that heterologous expression of Cav-1 in HC11 cells inhibits prolactin-induced activation of a STAT5a-responsive promoter by blocking STAT5a phosphorylation and DNA binding activity. Moreover, we demonstrate that Jak-2 cofractionates with caveolar membrane domains and coimmunoprecipitates with Cav-1 in mammary gland lysates. If Cav-1 normally functions as a suppressor of cytokine signaling in the mammary gland, then Cav-1 null mice should show premature development of the lobuloalveolar compartment because of hyperactivation of the prolactin signaling cascade via disinhibition of Jak-2. In accordance with this prediction, we demonstrate that Cav-1 null mice display accelerated lobuloalveolar development and precocious lactation. Furthermore, Cav-1-deficient mammary glands prematurely express milk proteins during pregnancy because of hyperactivation of STAT5a, and prolonged STAT5a phosphorylation was noted in Cav-1-deficient mammary glands during involution. Premature activation of the p42/44 MAPK cascade was also observed in Cav-1 null mammary glands during pregnancy.

Recently, Lindeman *et al.* (2001) demonstrated that SOCS1-deficient mice exhibited accelerated lobuloalveolar development and precocious lactation. As in our findings with Cav-1, recombinant expression SOCS1 in SCp2 mammary epithelial cells inhibited prolactin-dependent expression of β -casein. However, the precocious mammary development in SOCS1-deficient mice was not accompanied by

STAT5a hyperactivation during pregnancy (Lindeman *et al.*, 2001). This suggests that alternative unknown mechanisms of regulating Jak-2/STAT5a signaling remain intact in the SOCS1-deficient mice.

Here, we propose that Cav-1 functions in concert with SOCS1: 1) to fine-tune prolactin responses in the mammary gland and 2) to prevent the inappropriate onset of lactation during pregnancy. Whereas SOCS1 acts in a negative feedback loop to blunt Jak/STAT responses (Naka *et al.*, 1999), Cav-1 may function in a feed-forward manner (Park *et al.*, 2001). This dual regulation would allow for exquisite control of Prl-R signaling. As prolactin levels increase during pregnancy, in response, Cav-1 expression steadily declines (Park *et al.*, 2001), thereby allowing for a gradual induction of Prl-R signaling (Figure 8). Once Cav-1 expression is sufficiently down-regulated, lactation is induced and SOCS1 acts independently to modulate Jak-2/STAT5a signaling.

The development of premature lactation in both Cav-1 and SOCS1-deficient mice indicates that neither regulator alone is sufficient to fully suppress Jak-2/Stat5a signaling. Therefore, the coordinated action of Cav-1 and SOCS1 is necessary for appropriate mammary development during pregnancy and lactation. Cav-1-deficient mammary glands display accelerated lobuloalveolar development with STAT5a hyperactivation during pregnancy. In contrast, SOCS1-deficient mice exhibit STAT5a hyperactivation only during lactation (Lindeman *et al.*, 2001). From these observations, it can be inferred that Cav-1 inhibits Jak-2/STAT5a signaling during pregnancy, whereas SOCS1 acts during lactation. In support of this notion, the level of STAT5a hyperactivation seen in pregnant Cav-1 knockout mice is reduced with the onset of lactation, presumably because of the upregulation of SOCS1 (Figure 6B).

Furthermore, SOCS1-deficient mice exhibit more abundant milk production than wild-type mice, whereas Cav-1 knockout mammary glands do not produce more milk, but

rather have an earlier onset of milk production. These findings also implicate Cav-1 as a negative regulator of Jak-2/STAT5a signaling during pregnancy. Therefore, it is conceivable that the physiological trigger for the commencement of lactation is the downregulation of Cav-1 expression.

STAT5a is readily dephosphorylated as early as the first day of involution. Because Cav-1 is markedly up-regulated at the beginning of involution, it is possible that Cav-1 may also serve as a brake to turn off Jak-2 kinase activity. With the loss of Cav-1 upregulation during involution in Cav-1 null mice, Jak-2 activity continues unabated, leading to a prolonged activation of STAT5a. Therefore, it appears that expression of Cav-1 during pregnancy and involution are critical for maintaining the developmental borders of the mammary gland, i.e., the onset of lactation and involution.

The onset of lactation has been associated with the postpartum fall in serum estrogen and progesterone, with a concomitant increase in prolactin levels (Hennighausen and Robinson, 1998). Yet, how the decline of estrogen and progesterone levels signals the initiation of lactation remains unclear. The ability of estrogen and progesterone to act synergistically to up-regulate Cav-1 expression in hTERT-HME1 cells provides a potential mechanism by which the induction of lactation is regulated. Once estrogen and progesterone levels fall postpartum, prolactin can act without restriction to fully down-regulate Cav-1 and therefore trigger the induction of lactation. After Cav-1 expression is down-regulated, SOCS1 becomes the sole regulator of Jak-2/STAT5a signaling, and milk production ensues.

A balance between positive and negative regulators is critical for the stage-appropriate development of the mammary gland. As demonstrated by Cav-1 deficiency and SOCS1 deficiency, loss of either of these negative regulators leads to a profound defect in the orchestration of lobuloalveolar outgrowth and differentiation. Lactation imposes a considerable metabolic strain on the mother. In fact, in some species, the nutritional requirements of the mammary gland during lactation may exceed those of the rest of the organism. This incredible energy demand reinforces the need for tight regulation of the onset and termination of lactation. As such, the mammary gland uses a complex interplay of steroid, peptide, and growth hormones to modulate various positive and negative regulators of the prolactin signaling cascade.

Future studies will be needed to address the possible redundancy between Cav-1 and SOCS1 in the context of mammary gland development and lactation. In this regard, it would be interesting to analyze the phenotype of SOCS1/Cav-1 double-knockout mice. However, this may be technically difficult, because SOCS1-deficient mice exhibit neonatal lethality and require a second deletion of the interferon- γ (IFN- γ) gene to phenotypically rescue their viability (Lindeman *et al.*, 2001). Thus, the generation of a SOCS1/INF- γ /Cav-1 triple-knockout mouse would be required.

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