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Tissue-Specific Induction of SOCS Gene Expression by PRL

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The mechanisms whereby tissue sensitivity to PRL is controlled are not well understood. Here we report that expression of mRNA and protein for members of the SOCS/CIS/JAB family of cytokine signaling inhibitors is increased by PRL administration in ovary and adrenal gland of the lactating rat deprived of circulating PRL and pups for 24 h but not in mammary gland. Moreover, suckling increases SOCS mRNA in the ovary but not in the mammary gland of pup-deprived rats. Deprivation of PRL and pups for 48 h allows the mammary gland to induce SOCS genes in response to PRL administration, and this is associated with a decrease in basal SOCS-3 mRNA and protein expression to the level seen in other tissues, suggesting that SOCS-3 induced refractoriness related to filling of the gland. In reporter assays, SOCS-1, SOCS-3, and CIS, but not SOCS-2, are able to inhibit transactivation of the STAT 5-responsive β-lactoglobulin promoter in transient transfection assays. Moreover, suckling results in loss of ovarian and adrenal responsiveness to PRL administered 2 h after commencement of suckling, as determined by STAT 5 gel shift assay. Immunohistochemistry was used to localize the cellular sites of SOCS-3 and CIS protein expression in the ovary and adrenal gland. We propose that induced SOCS-1, SOCS-3, and CIS are actively involved in the cellular inhibitory feedback response to physiological PRL surges in the corpus luteum and adrenal cortex during lactation, but after pup withdrawal, the mammary gland is rendered unresponsive to PRL by increased levels of SOCS-3. (*Endocrinology* 142: 5015–5026, 2001)

PRL IS A class 1 cytokine that has more than 300 documented actions across many target tissues (1). These are thought to be initiated by hormone-induced receptor homodimerization, which results in the activation of receptor-associated Janus kinase (JAK) 2 and fyn tyrosine kinases (2). These activated kinases initiate a number of signaling pathways, including activation of the latent cytoplasmic transcription factors STAT 1, 3, 5a, and 5b by tyrosine phosphorylation and subsequent dimerization (3). Other PRL-activated signaling pathways include the MAPK cascade, increase of cellular free calcium levels, and activation of phospholipase Cγ (1, 2, 4, 5). Termination of the tyrosine phosphorylation signal involves phosphatase action at the level of JAK 2 and the STATs (6), although the phosphatase(s) are not well defined. Regulation of target tissue sensitivity to PRL has long been thought to be controlled through receptor expression, for example, through up-regulation of mammary gland receptor expression at parturition in the rat (7), with a corresponding decrease in receptor expression in the corpus luteum, which is thought to decrease progesterone secretion (8). Recently, PRL and PRL receptor knockout mice have been created, and these have emphasized the important role of PRL in mammary alveolar epithelium development, ovarian function, and embryo implantation (9, 10). The importance of the STAT 5s, particularly STAT 5a, in mammary alveolar and ductal development, was also recently demonstrated by specific STAT 5a and 5b knockout (11, 12), which confirmed the view from *in vitro* studies that PRL activation of STAT 5a is a central feature of its role in mammary alveolar epithelial development and lactation (1, 4, 13). Tyrosine phosphorylation of the PRL receptor, demonstrable both *in vitro* and *in vivo* (14), promotes STAT 5 activation by providing SH2 docking sites, particularly Y580 of the full length receptor (15).

Our earlier study (14) indicated that the rabbit mammary gland is refractory to PRL-induced tyrosine phosphorylation of both the receptor and JAK 2 unless the rabbit is deprived of PRL with a dopamine agonist for at least 24 h. This, and the observation that removal of residues 322–333 within the cytoplasmic domain increases PRL-induced activation of a milk protein promoter (16), led us to propose that a PRL-inducible inhibitory protein was bound to the receptor that suppressed JAK 2 activation (14). Recently, a family of cytokine-inducible inhibitors of cytokine signaling has been reported (17–21), although the first member of the family, CIS (cytokine-inducible SH2-containing protein), has been known for several years (22). These SOCS (suppressor of cytokine signaling) proteins appear to act as feedback inhibitors of signaling for a wide range of cytokines that act through the JAK/STAT (signal transducer and activator of transcription) pathway (20). They possess a variable N-terminal region preceding an SH2 domain, followed by a conserved C-terminal region of approximately 40 residues known as the SOCS box, which is thought to regulate proteolysis (23). This motif is found in 20 proteins in five structural classes (24). SOCS-1 binds directly to JAK kinases, in-

Abbreviations: CAT, Chloramphenicol acetyl transferase; CIS, cytokine-inducible SH2-containing protein; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; HRP, horseradish peroxidase; JAK, Janus kinase; oPRL, ovine PRL; PMSF, phenylmethylsulfonyl fluoride; SOCS, suppressor of cytokine signaling, STAT, signal transducer and activator of transcription.

cluding JAK 2 and Tec kinases, inhibiting their catalytic activity (23, 25). CIS, on the other hand, binds to tyrosine-phosphorylated β-chain of IL-3, erythropoietin (22), and GH receptors (26), inhibiting their proliferative actions and, in the case of the GH receptor, increasing proteasomal degradation of the receptor signaling complex (27). SOCS-3 was shown to be induced by GH and leptin and to inhibit STAT 5-mediated transactivation of the serine protease inhibitor 2.1 promoter by GH by binding to membrane-proximal phosphotyrosine residues in the GH receptor (26, 28, 29). These and other *in vitro* studies of SOCS indicate that they act in a classic feedback loop manner to inhibit the action of tissue-relevant cytokines. This is supported *in vivo* by the recent SOCS-1 knockout in which the thymus was smaller, a loss of mature B cells was observed, and the mice showed stunted growth and died before weaning with fatty degeneration of the liver (30). These symptoms could be explained by unopposed action of interferon-γ overexpression, a cytokine that activates SOCS expression (17, 30). On the other hand, CIS-overexpressing transgenic mice exhibit impaired mammary gland development and growth retardation, although CIS knockout mice have no obvious phenotype (3, 31). SOCS-3 knockout and overexpressing transgenic mice are both embryonic lethal, owing to the disruption of hepatic hematopoiesis (31). Recently, SOCS-2 knockout mice were found to exhibit a phenotype resembling GH-overexpressing transgenic mice (32).

The aim of this study was to assess the involvement of SOCS genes in PRL action *in vivo* in major PRL target tissues. We have used PRL-suppressed lactating rats as our *in vivo* model, because we have shown that they respond to PRL injection with tyrosine phosphorylation of both the receptor and JAK 2 and with STAT 5 activation (33). We present evidence for the induction of expression of SOCS genes by PRL *in vivo*, for their involvement in PRL signaling, and for differential sensitivity of PRL target tissues to induction of these suppressors of signaling.

Materials and Methods

Materials

Ovine PRL (oPRL) was a gift of the National Hormone and Pituitary Program (Baltimore, MD). pBOS-based expression vectors for SOCS 1–3 and CIS have been described (17). Rabbit PRL receptor cDNA in pECE and the chloramphenicol acetyl transferase (CAT) reporter construct for β-lactoglobulin have been described as well (6). STAT 5a expression plasmid was a generous gift of Dr. B. Groner (Tumor Biology Center, Freiburg, Germany). Goat anti-SOCS-3 (sc 7009) and anti-CIS (catalog no. sc 1529) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) along with the respective blocking peptides. Rabbit anti-CIS antibody (34) was a generous gift of Prof. A. Yoshimura (Institute of Life Science, Kurume, Japan). Rabbit anti-SOCS-3 antibody was raised against murine SOCS-3. Bromocriptine was purchased from Sigma (St. Louis, MO).

Animals

Twenty-four or 48 h before experiments, pups were removed from 5- to 10-day lactating Wistar rats, and the mothers were injected sc with 1.5 mg/kg bromocriptine dissolved in saline-ethanol (7:3) to block endogenous PRL secretion (33). Injections were repeated every 12 h. Then, 24 or 48 h after the first injection, rats were injected ip with 0.25 mg of oPRL in saline containing 10 mM $NaHCO_3$ or vehicle alone. Rats were killed 0, 30, 60, 120, 240, or 440 min after PRL injection. A separate group of animals was removed from the pups and killed immediately.

For the initial suckling experiments (four per group), pups were removed for 16 h from lactating mothers, and then mothers were put back with the pups for 2 h before being killed. A control group was kept separate from the pups before being killed. To study the effect of suckling on subsequent response to PRL administration, four groups were used. Two groups ("suckled" groups) were put back to suckle their pups for 1 h and then the pups were removed for a further 1 h: one group was then given oPRL (0.25 mg, as above), while the other group was injected with saline vehicle. After a further 30 min, these rats were killed and their tissues were processed. With the other two groups ("nonsuckled" groups), after the 16-h pup deprivation period, one group was injected with oPRL (0.25 mg, as above) 30 min before being killed, while the other group was injected with saline vehicle.

In all cases, the mammary glands, ovaries, liver, and adrenal glands were quickly excised, snap frozen in liquid nitrogen, and stored at −80 C until processing for Northern, electrophoretic mobility shift assay (EMSA), or Western blot analysis. Tissues were fixed in 4% buffered paraformaldehyde for immunohistochemistry. These experiments were authorized by the University of Queensland institutional animal ethics committee according to National Health and Medical Research Council (Australia) guidelines.

Northern hybridization

Total RNA was isolated from tissues using TRIzol Reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer's protocol. Aliquots of 20 μg of total RNA were denatured and subjected to electrophoresis on a 1.2% agarose, 11% formaldehyde gel and then transferred to a HyBond N membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). After UV cross-linking and then baking for 30 min at 80 C, the membranes were prehybridized in NorthernMax prehyb/hyb buffer (Ambion, Inc., Austin, TX) for 4 h at 50 C. ^{32}P-labeled cDNA probes for SOCS-1, -2, -3, or CIS [prepared by random prime labeling using the Rediprime II system (Amersham Pharmacia Biotech, Uppsala, Sweden)] were then added, and the membranes were hybridized at 50 C for 16 h. After this, the membrane was washed twice in 2× SSC (30 mM sodium citrate, 300 mM sodium chloride, pH 7.0), 0.1% SDS at 65 C for 30 min. After autoradiography at −70 C, all blots were stripped and rehybridized with a ^{32}P-end-labeled oligomer probe specific for 18S rRNA to determine loading. SOCS mRNA expression was then normalized to 18S RNA expression from densitometer scans using the Bio-Rad Laboratories, Inc. (Hercules, CA) GS-700 densitometer with Molecular Analyst software (Bio-Rad Laboratories, Inc.).

EMSA

For the preparation of nuclear extracts (35), tissues were homogenized with a Polytron homogenizer and then incubated for 15 min in 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 20 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, and 2 μg/ml aprotinin and leupeptin (pH 7.9) on ice. Cells were lysed by the addition of Nonidet P-40 (Schleicher & Schuell, Inc., Keene, NH) to 0.6% and microfuged at 13,000 rpm for 30 sec. Nuclear pellets were resuspended in 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 20 mM NaF, 0.5 mM PMSF, 2 μg/ml aprotinin and leupeptin, and 20% glycerol. Extracts were then divided into aliquots and stored at −80 C.

The complementary strands of Stat 5 oligonucleotide probe were obtained from Pacific Oligos (Lismore, New South Wales, Australia) and contained the sequence 5′-AGATTTCTAGGAATTCAATCC-3′ [binding sites are *underlined* (4)]. Annealed, double stranded oligonucleotides were 5′-end labeled with T4 polynucleotide kinase and purified on a 10% polyacrylamide gel.

To perform the gel shift assay, 5 μl of nuclear extract was added to a binding mixture (22 μl) containing 180 μg/ml BSA, 90 μg/ml poly(dI-dC), 12 mM HEPES (pH 7.9), 12% glycerol, 0.12 mM EDTA, 0.9 mM $MgCl_2$, 0.6 mM DTT, 0.6 mM PMSF, 120 mM KCl, and 1.2 μg/ml aprotinin and leupeptin (35). This binding mixture was incubated with or without 3 μl of STAT 5b antiserum (Santa Cruz Biotechnology, Inc.; catalog no. sc 835), which recognizes both STAT 5a and 5b, for 1 h on ice and for a further 30 min after addition of 0.5–1 ng of labeled probe. Samples were then electrophoresed on 6% polyacrylamide, 1× TBE (90 mM Tris-

borate-20 mM EDTA) gels at 4 C. Gels were then dried and exposed to Fuji Photo Film Co., Ltd. (Tokyo, Japan) RX film at −70 C. Equal loading of the nuclear extracts on these gels was confirmed by a parallel run with an octamer-binding protein-1 probe as previously described by Clarkson *et al.* (35).

Immunohistochemistry

For localization of SOCS-3 and CIS immunoreactivity in ovary and adrenal gland, 5-μm paraffin sections were dewaxed and rehydrated, pretreated with 3% H_2O_2, and then blocked with 10% normal rabbit serum. Sections were incubated with goat anti-CIS (4 μg/ml; Santa Cruz Biotechnology, Inc.; cat sc 1529), goat anti-SOCS-1 (4 μg/ml; Santa Cruz Biotechnology, Inc.; cat sc 7006), or goat anti-SOCS-3 (4 μg/ml; Santa Cruz Biotechnology, Inc.; cat sc 7009) overnight at 4 C. After washing, sections were incubated with biotin-labeled rabbit antigoat IgG (Zymed Laboratories, Inc., San Francisco, CA; cat 50-232) and horseradish peroxidase (HRP)-streptavidin (Zymed Laboratories, Inc.; cat 85–9943, with dilutions as recommended by manufacturer) for 1 h at 20 C. Immunoreactivity was localized with 3-amino-9-ethylcarbazole substrate (Zymed Laboratories, Inc.; cat 85-9943). For negative controls, primary antibodies preincubated for 2 h at 20 C with SOCS-3 and CIS blocking peptides (20 μg/ml; Santa Cruz Biotechnology, Inc.; cat sc 7009p and 1529p, respectively) were used. Rabbit antibodies to CIS (a gift of Prof. A. Yoshimura; dilution, 1:500) and SOCS-3 (dilution, 1:50) were used to confirm the immunoreactive distribution found with the goat antibody, with in this case normal rabbit serum as the primary antibody control (see above).

Mammary gland paraffin sections was dewaxed, rehydrated, and pretreated with H_2O_2 as described above. Sections were blocked with 10% normal horse serum followed by 1:20 rabbit anti-CIS antiserum (a gift from A. Yoshimura), 1:20 rabbit anti-SOCS antiserum, or 1:20 nonimmune rabbit serum (as negative control) overnight at 4 C. Thereafter, sections were incubated with 1:200 biotinylated donkey antirabbit IgG (cat RPN1004; Amersham Pharmacia Biotech, Buckinghamshire, UK) for 2 h at room temperature and followed by 1:200 streptavidin-biotinylated HRP complex (cat RPN 1051; Amersham Pharmacia Biotech, Buckinghamshire, UK) for 2 h at room temperature. The signal was visualized by incubation with DAB substrate for 5 min. Sections were counterstained with hematoxylin and dehydrated before mounting.

Western blotting

This was undertaken using the protocol described previously (14) with minor modifications designed to allow the use of cytosol directly. Frozen ovary was homogenized in 5 volumes of cytosol buffer [40 mM Tris-HCl, 5 mM EGTA, with complete protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany; cat 1697498) pH 7.6] and then centrifuged at 15,000 rpm in a microfuge for 15 min at 4 C. To 20 μl of cytosol was added 10 μl of 3× Laemmli sample buffer, and the solution was boiled for 3 min before loading on a 12% (3.3%C) (cross-link) Laemmli gel (14). After semidry transfer in 20% methanol in Laemmli electrophoresis buffer, membranes were blocked with 2% BSA (ICN, Costa Mesa, CA; cat 160069) in 0.1% Tween in Tris-buffered saline (14). The blots were incubated overnight at 4 C with goat anti-SOCS-3 and anti-CIS at 1:250. HRP-labeled antirabbit and antigoat antisera were then used for visualization by enhanced chemiluminescence.

For mammary gland, samples from 48-h bromocriptine-treated rats and from lactating mothers with or without pup withdrawal for 16 h were prepared as described above except that tissues were homogenized in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5) with complete protease inhibitor cocktail (Roche Molecular Biochemicals; cat 1697498). Lysates were then boiled in Laemmli sample buffer before being centrifuged. DNA in the supernatant was sheared by passing through a 25-gauge needle 10 times before storage. One hundred micrograms of lysate protein (approximately 2 μl of lysate) was then Western blotted as described above. After transfer, nitrocellulose membranes were blocked with Tris-buffered saline (pH 8) containing 5% teleostean gelatin (Sigma; cat G 7765), 0.1% Tween 20 and probed with goat anti-SOCS-3 antibody (1:250; Santa Cruz Biotechnology, Inc.) at 4 C overnight. Thereafter, membranes were incubated with HRP-conjugated rabbit antigoat antibody (Pierce Chemical Co., Rockford, IL) at 1:10,000 for 1 h at room temperature followed by development with Western blot Renaissance chemiluminescence reagent plus (New England Nuclear Life Science Products, Boston, MA).

Cell transfection

Monkey kidney COS-1 cells were maintained in DMEM supplemented with 10% Serum Supreme and 5 μg/ml gentamycin. The cells were seeded onto six-well plates, cultured to 40% confluence, and then serum starved overnight in GC3 medium [Ham's F12:DMEM (1:1) containing 1× insulin-transferrin-sodium selenite media supplement (Sigma), 1× nonessential amino acids (Life Technologies, Inc.), and 5 μg/ml gentamycin]. For each well, 90 μl of transfection reagent/plasmid mixture containing 2 μg of rabbit PRL receptor expression plasmid, 1 μg of β-lactoglobulin CAT reporter, 0.4 μg of Stat 5a expression plasmid, 0.2 μg of β-galactosidase reporter, 0.5 μg of SOCS-1, SOCS-2, SOCS-3, or CIS expression plasmid, and 20 μl of (*N*-[1-(2,3-dioleoyl)propyl]-*N,N,N*-trimethylammonium methyl-sulfate liposomal transfection reagent (Roche Molecular Biochemicals) in HBS (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM dextrose, pH 7.05) was used (6). The transfection mixture was mixed gently and incubated for 15 min at 20 C before adding it to each well in 2 ml of GC3 medium. After incubation for 7 h, the medium was replaced and the cells were left for 16 h. Cells were treated with 500 ng/ml oPRL for 24 h in GC3 medium. The cells were then harvested for measurement of CAT and β-galactosidase activities.

CAT assays

CAT assay was performed as described by Downes *et al.* (36). The cells were lysed by freezing and thawing three times in 0.25 M Tris-HCl, pH 7.8, on a dry ice/ethanol bath. Aliquots of cell supernatant were incubated with a mixture of 0.2 mCi of [^{14}C]chloramphenicol, 7 mM acetyl coenzyme A, and 0.25 M Tris-HCl, pH 7.8, at 37 C for 6 h. The chloramphenicol and its acetylated forms were then extracted with 1 ml of ethyl acetate and resolved by running the extract on Silica gel TLC plates in chloroform/methanol (19:1). Plates were analyzed with an AMBIS β-scanner. Results were normalized to β-galactosidase expression as described (San Diego, CA) (6).

Statistical analysis

Multiple comparisons were undertaken by ANOVA using Tukey's post hoc analysis.

Results

PRL induces SOCS mRNA in vivo

Administration of oPRL to lactating rats treated with bromocriptine for 24 h resulted in rapid induction of mRNAs for SOCS-1, SOCS-2, SOCS-3, and CIS in the ovary, commencing at 30 min, reaching a maximum at 60–120 min, and returning close to baseline by 440 min. SOCS-2 mRNA was induced more slowly, and SOCS-3 mRNA decreased more rapidly than those of other SOCS (Fig. 1). In the case of the adrenal gland, rapid induction of SOCS-3 and CIS mRNA was seen, with no significant response for SOCS-2 and SOCS-1 mRNA. The induction of SOCS-3 mRNA was even more transient than that seen with the ovary (Fig. 2). The liver did not respond consistently to PRL administration. The mammary gland did not show induction of any SOCS mRNAs in response to PRL administration to these bromocriptine-treated, 24-h pup-deprived mothers. In all tissues, no induction of SOCS mRNA was seen 60 min after injection of saline, demonstrating that the induction we observed is not stress related.

Western blot evidence for increased expression of SOCS proteins

We were able to show increased SOCS-3 and CIS protein expression in cytosolic extracts of ovary in response to PRL

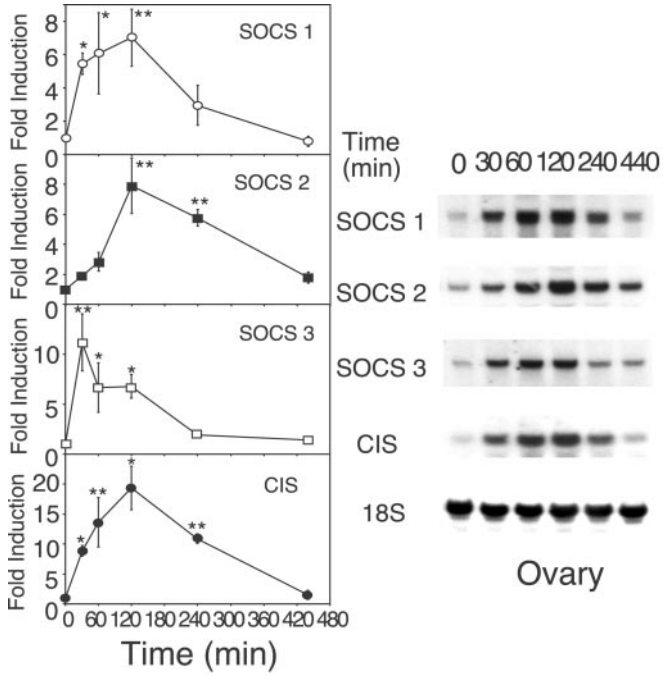

FIG. 1. Time course of induction of SOCS-1, SOCS-2, SOCS-3, and CIS mRNA expression in the ovary in response to PRL administration. Lactating rats deprived of pups and given bromocriptine for 24 h were injected with vehicle or 0.25 mg oPRL in vehicle and then killed at the indicated times after injection. Total RNA was extracted from the tissues, and Northern analyses were performed with 20 μg of total RNA per lane. Blots were stripped and reprobed for 18S to ensure equal loading. Further details are given in *Materials and Methods*. *Bar charts* show the mean induction of SOCS mRNA ± SEM (n = 6 for the control, n = 3 for other groups; *, $P < 0.05$; **, $P < 0.01$) normalized with 18S rRNA levels and expressed as fold induction of control. The *right panel* shows a representative Northern blot.

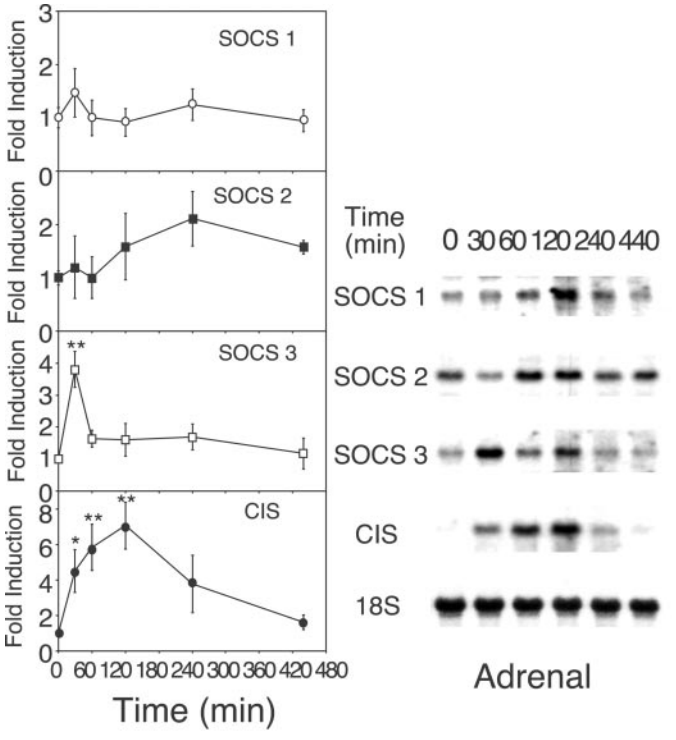

FIG. 2. Time course of induction of mRNAs for SOCS family members in response to PRL administration in the adrenal gland. Procedures were as described for Fig. 1, except that 18 μg of total RNA was used.

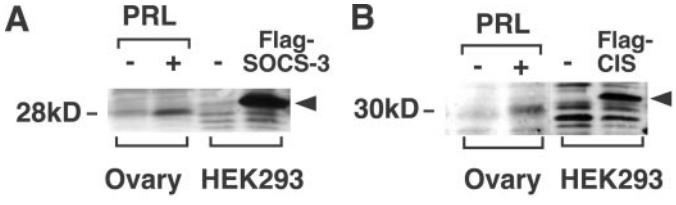

FIG. 3. PRL induces SOCS-3 and CIS protein expression in ovary. Total cytosol lysates were prepared from ovaries of bromocriptine-treated rats injected with PRL or vehicle for 120 min before being killed. Lysates were electrophoresed on SDS-PAGE, immunoblotted with goat anti-SOCS-3 (A) and anti-CIS (B) antibody followed by HRP-conjugated antigoat antiserum, and visualized by chemiluminescence. HEK293 cells and HEK293 cells transiently transfected with FLAG-tagged SOCS-3 or CIS vectors (*arrowheads*) were used as negative and positive controls, respectively. Molecular mass positions of the endogenous SOCS-3 and CIS are shown; FLAG fusion proteins carry an additional molecular mass of 2 kDa.

administration. In Fig. 3, it can be seen that an immunoreactive protein band migrating just below FLAG-labeled CIS and SOCS-3 (expressed in HEK293 cells) increased in intensity 2 h after PRL injection.

Cellular localization of SOCS proteins

We were able to show the cellular location of CIS and SOCS-3 immunoreactivity in the ovary and adrenal glands (Fig. 4), but we were unable to demonstrate increased immunoreactivity in response to PRL administration. For both tissues, immunoreactivity was abolished by pretreatment with the peptide used for immunization. This could not be shown for SOCS-1 immunoreactivity (not shown). In the adrenal gland, immunoreactivity was present in the cortex, particularly in the zona fasciculata, but elements within medullary tissue were also immunoreactive. In the ovary, immunoreactivity was strong in some corpora lutea cells but absent from follicular granulosa cells. There was weak staining in stroma and in the endothelium of blood vessels. Other ovarian components were not immunoreactive. Localization of SOCS-3 and CIS immunoreactivity in the ovary was confirmed separately with relevant rabbit antibodies, and these gave clear cellular localization within luteal cells and a stronger immunoreaction (Fig. 5, e and f). These rabbit antibodies were able to detect cellular immunoreactive CIS and SOCS-3 in ducts and alveolar epithelial cells of the lactating mammary gland (Fig. 5, a–c).

Induction of SOCS gene expression in response to suckling

To establish if suckling associated with physiological levels of PRL stimulus was able to induce SOCS mRNA expression, pups were removed from mothers overnight (16 h) and then returned to their mothers. Two hours after suckling, tissues were removed and processed for Northern analysis. Clear induction of SOCS-1, SOCS-2, and CIS mRNAs was seen in the ovary (Fig. 6A). Again, no induction of any SOCS mRNA was apparent in the mammary gland, including that for SOCS-3 (Fig. 6B).

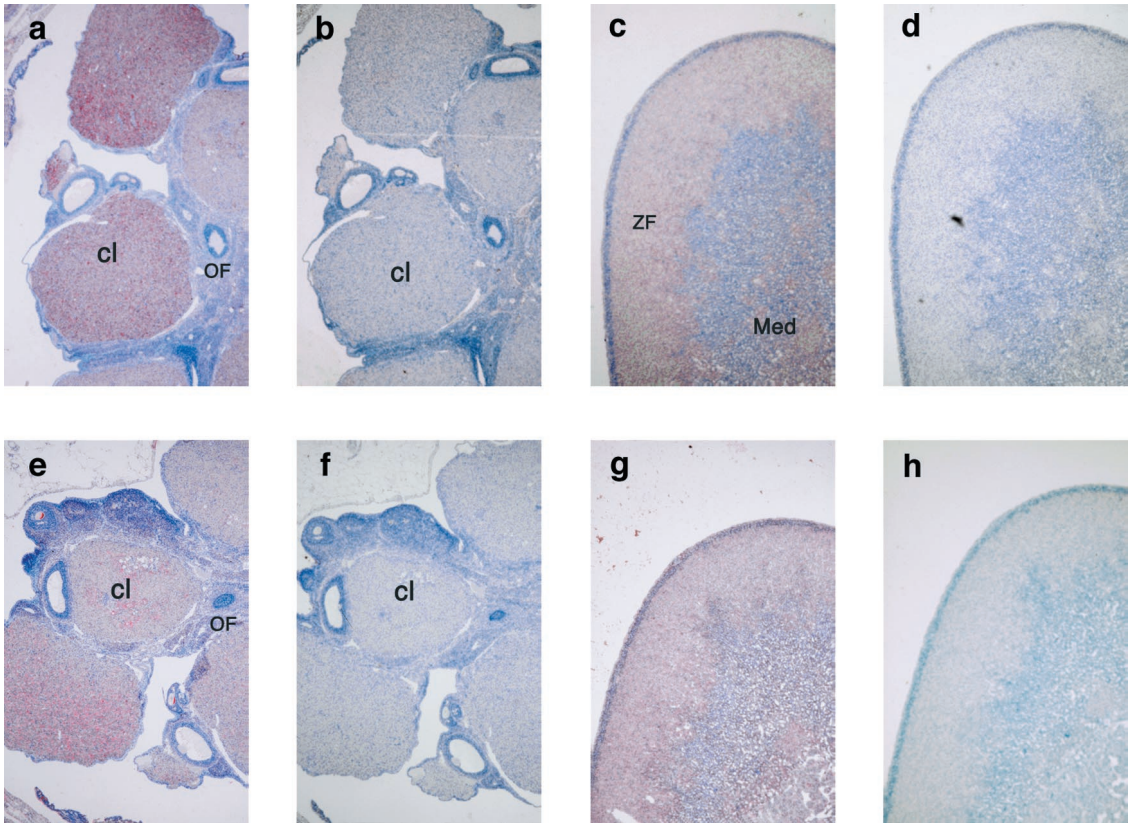

FIG. 4. CIS and SOCS-3 are predominantly expressed in the corpus luteum in ovary and in the adrenal cortex in adrenal gland. Tissues taken from 5- to 7-day lactating rats were fixed and processed as described in *Materials and Methods*. Briefly, paraffin sections were incubated overnight with goat anti-CIS or anti-SOCS-3 antibody with or without preincubation with the appropriate blocking peptide, followed by incubation with biotin-conjugated antigoat IgG and streptavidin-biotinylated HRP, and development with 3-amino-9-ethylcarbazole substrate counterstained with hematoxylin. Ovary was immunostained *red* with SOCS-3 antibody without (a) but not with (b) preincubation with SOCS-3 blocking peptide. Adrenal gland was stained with SOCS-3 antibody without (c) but not with (d) preincubation with SOCS-3 blocking peptide. Ovary was stained with CIS antibody without (e) but not with (f) preincubation with CIS blocking peptide. Adrenal gland was stained with CIS antibody without (g) but not with (h) preincubation with CIS blocking peptide. Definitive immunoreactivity for CIS and SOCS-3 could not be obtained with these antibodies in mammary gland sections.

The mammary gland can respond to PRL, as shown by STAT 5 activation

To determine whether the lactating rat mammary gland can respond to PRL, we measured active STAT 5 by EMSA in nuclear extracts from animals deprived of PRL for 24 h (bromocriptine treated) and then injected with saline or PRL. Fig. 7 shows strong induction of STAT 5 element binding in response to PRL injection in both the ovary and mammary gland. The identity of the bandshift protein appears to be STAT 5, because the band was supershifted with a STAT 5 antibody but not a STAT 4 antibody. In a previous study, we showed that STAT 1 and STAT 3 oligonucleotides do not compete for this band in rat mammary nuclear extracts (33).

SOCS genes can be induced by PRL in the mammary gland 48 h after pup removal

Because there is a block to SOCS gene induction by PRL in lactating mammary gland after 24-h withdrawal of PRL and pups, we investigated SOCS gene induction after 48 h of the same treatment and found that this did render the mammary gland responsive to PRL administration, with substantial induction of SOCS-1, SOCS-3, and CIS but not SOCS-2 mRNAs (Fig. 8). This was associated with a decline in control (no stimulation) levels of SOCS-3 but not of SOCS-1, SOCS-2, or CIS mRNA expression (Fig. 9). Importantly, unstimulated SOCS-3 mRNA was substantially increased in 24-h-deprived lactating mammary gland compared with other tissues, whereas the level of SOCS-3 mRNA in non-bromocriptine-treated rats kept with their litters was very low. Thus, there is a correlation between unstimulated or basal SOCS-3 mRNA and the ability of PRL to induce SOCS gene expression in the mammary gland.

We verified that the SOCS 3 mRNA was processed to protein in the 48-h PRL- and pup-deprived mammary gland (Fig. 10). Importantly, this figure also shows that unstimulated/basal SOCS 3 protein is significantly increased at 16 h compared with that in normal suckling animals.

Functional consequences of SOCS expression on PRL signaling

We next determined if SOCS proteins are able to influence PRL-dependent transactivation of the β-lactoglobulin gene, encoding an important milk protein. Using transient cotransfection assays in COS-1 cells with a CAT reporter, we found

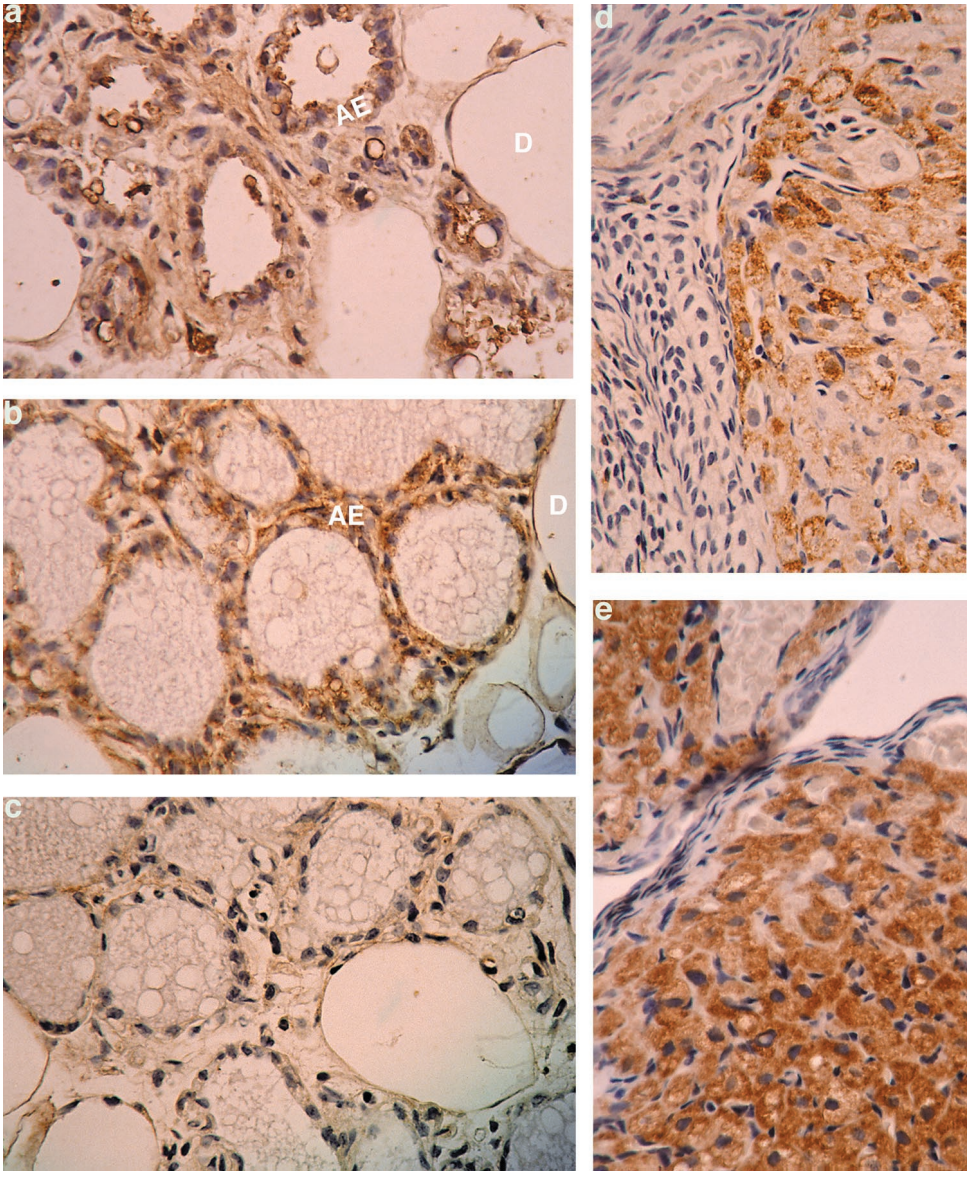

FIG. 5. CIS and SOCS-3 proteins are expressed in mammary gland and ovary from 5-day lactating rats. Ovary or mammary gland tissue was taken from animals after 48 h of bromocriptine and PRL treatment. Paraffin sections were immunostained with rabbit anti-CIS and anti-SOCS-3 antibody (see *Materials and Methods*), followed by incubation with biotinylated donkey antirabbit IgG and streptavidin-biotinylated HRP complex. The signal was visualized with diaminobenzidine substrate. Mammary epithelial cells were immunostained *brown* when incubated with anti-CIS antibody (a) or anti-SOCS-3 antibody (b) but not with preimmune rabbit serum (c). Ovarian corpus luteal cells were similarly immunostained by these anti-CIS (d) and anti-SOCS-3 (e) antibodies compared with antigoat antibodies (Fig. 4).

that SOCS-1, SOCS-3, and CIS, but not SOCS-2, could abrogate PRL activation of the β-lactoglobulin promoter (Fig. 11).

Exposure to increased PRL secretion (suckling) decreases the ability of PRL injection to activate STAT 5

We determined if the ability of the ovary and adrenal gland to respond to PRL administration was influenced by previous exposure to suckling-induced PRL secretion. Using induction of STAT 5 binding activity in EMSA, we observed a marked decrease in responsiveness to the standard dose of PRL administered 2 h after 1 h of suckling (Fig. 12).

Discussion

This work shows that PRL is able to induce expression of members of the SOCS family in lactating rats, but there are tissue-specific differences in responsiveness to PRL and the suckling stimulus. Sites of SOCS protein expression were shown to correspond with those of PRL receptor expression as determined by immunohistochemistry (7, 37). Increase of plasma PRL by the physiological stimulus of suckling results in an increase of SOCS expression and refractoriness to a subsequent injection of PRL.

It is known that STAT 5a and STAT 5b both bind to multiple upstream regulatory elements in the CIS gene promoter (34, 38). These contribute to the induction of CIS mRNA by erythropoietin (34) and presumably by PRL, because, as we show here, PRL induces STAT 5a binding activity in both ovary and mammary gland *in vivo*, as also reported elsewhere (33, 39). Because in STAT 5b knockout mice GH is able to induce CIS or SOCS-2 mRNAs in virgin mammary tissue, it is likely that STAT 5a is the key STAT controlling CIS and SOCS-2 transactivation in the rodent mammary gland (40). In the case of the SOCS-3 promoter, which is likewise rapidly transactivated by a number of class 1 cytokines (20), its transcription is known to be regulated by a STAT 3 responsive element proximal to the transcription start site (41), and there

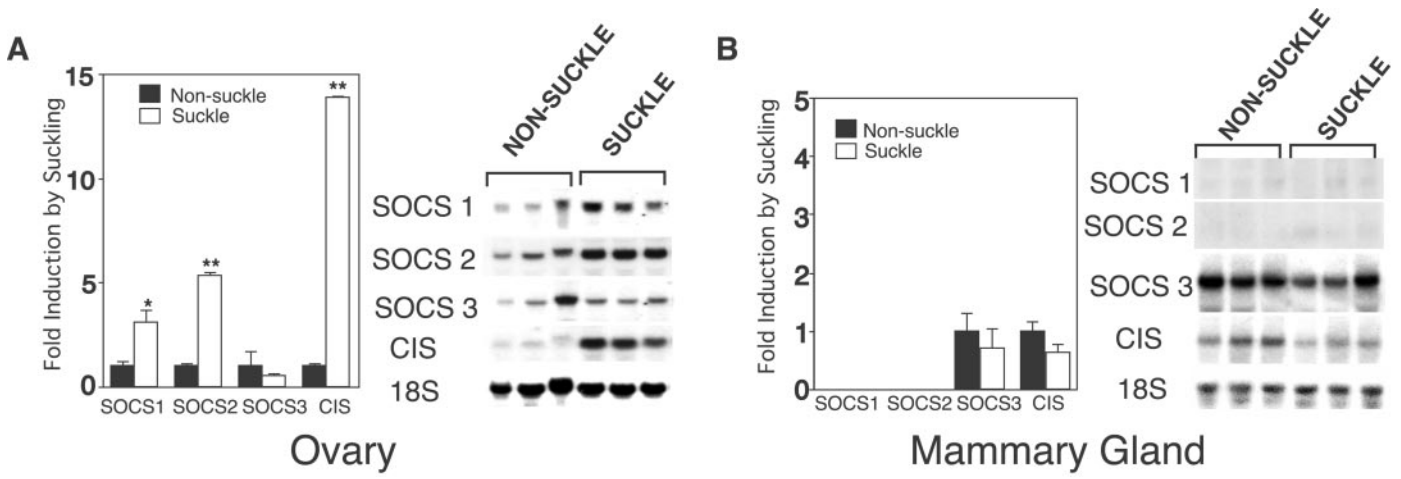

FIG. 6. Effect of suckling on SOCS-1, SOCS-2, SOCS-3, and CIS mRNA expression in ovary and mammary gland. Pups were removed from lactating rats for 16 h and then returned to the mothers for 2 h of suckling. A control group (nonsuckle) was left without returning the pups. Total RNA was extracted from the tissues, and Northern analyses were performed with 20 μg of total RNA per lane. Blots were stripped and reprobed for 18S to ensure equal loading. *Bar charts* show the mean fold induction of SOCS mRNA in ovary (A) and mammary gland (B) by suckling ± SEM (n = 3; *, $P < 0.05$; **, $P < 0.01$). *Right panels* show representative Northern blots of the data. SOCS-1 and SOCS-2 mRNA levels were nearly undetectable in mammary gland, whereas SOCS-3 and CIS mRNAs were not induced by suckling.

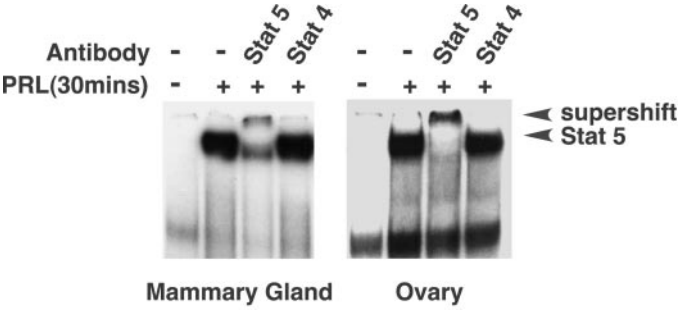

FIG. 7. EMSA with nuclear extracts from lactating mammary gland or ovaries from rats treated with or without PRL as described in Fig. 1. Extracts were incubated with a STAT 5 oligonucleotide probe and then run on a 6% polyacrylamide gel as described in *Materials and Methods*. PRL induced a band that could be supershifted by STAT 5 antiserum but not by STAT 4 antiserum.

is also good evidence that PRL induces STAT 3 activation in the rat ovary (42). SOCS-1 mRNA is also known to be induced by activated STAT 3 (19), which is consistent with SOCS-1 mRNA induction by PRL.

As indicated, the pattern of SOCS member response to PRL differs between rat tissues. In the ovary, all four SOCS mRNAs examined are induced by a high dose of PRL, although with the physiological suckling stimulus, only SOCS-1, SOCS-2, and CIS are induced. In the adrenal gland, where PRL influences steroid metabolism in the cortex, only CIS and SOCS-3 mRNAs are induced by PRL. In the liver, there was no consistent SOCS response to PRL, consistent with the difficulty in showing tyrosine phosphorylation and STAT activation in the rat liver (33). In the mouse liver, Pezet *et al.* (43) reported strong induction of SOCS-3 mRNA in response to PRL, which may relate to differences in the proportion of short and long form PRL receptors in the rat. In the 48-h PRL-deprived mammary gland (but not in the 24-h-deprived gland), SOCS-1, SOCS-3, plus CIS, but not SOCS-2 mRNAs, are induced. Thus, there is tissue specificity in the SOCS genes that are induced by PRL. In a survey of SOCS gene induction by cytokines, Aman and Leonard (20) concluded that CIS and SOCS-3 are induced by all 14 cytokines studied, whereas the induction of SOCS-1 and, to a lesser extent, SOCS-2 is more cytokine specific. Interestingly, the cytokine most closely related to PRL, GH, strongly induces SOCS-3 mRNA but induces other SOCS members only weakly. In mouse liver, as with PRL, SOCS-3 and CIS were the only SOCS members induced by GH (28). The selection of SOCS genes induced by PRL could be seen as an integrated response able to totally or selectively attenuate elements of the PRL signaling process. In the absence of significant SOCS-1 induction, it is likely that JAK 2 signaling would retain significant activity, whereas STAT 5 activation, and probably other signaling pathways dependent on PRL receptor association through SH2 domain interaction, would be selectively impaired by high CIS and SOCS-3 expression, because these presumably associate with the phosphorylated receptor in the same manner as with the IL-3, erythropoietin, and GH receptors (15, 22, 26).

Of particular interest in this study is the correlation between the unstimulated (control) level of SOCS-3 expression and the ability of the lactating mammary gland to respond to PRL at the level of gene induction. The gland was not able to respond to PRL (administered or suckling induced) 24 h after pup withdrawal, yet both the ovary and the adrenal gland were fully responsive, as indicated by the ability to induce SOCS genes. However, by 48 h after pup removal, the PRL response had returned, coincident with a decrease in unstimulated SOCS-3 level toward the baseline level seen in suckling mothers. We were also unable to obtain an induction of mammary acetyl coenzyme A carboxylase by PRL in 24-h pup-deprived mothers (not shown), although this is the most *in vivo* PRL-responsive gene in the rat mammary gland (44). Hormone resistance consequent to increased SOCS-3 has been reported for leukemia inhibitory factor-induced desensitization of the pituitary corticotroph (41), for hypothalamic resistance to leptin (29), for endotoxin-induced GH

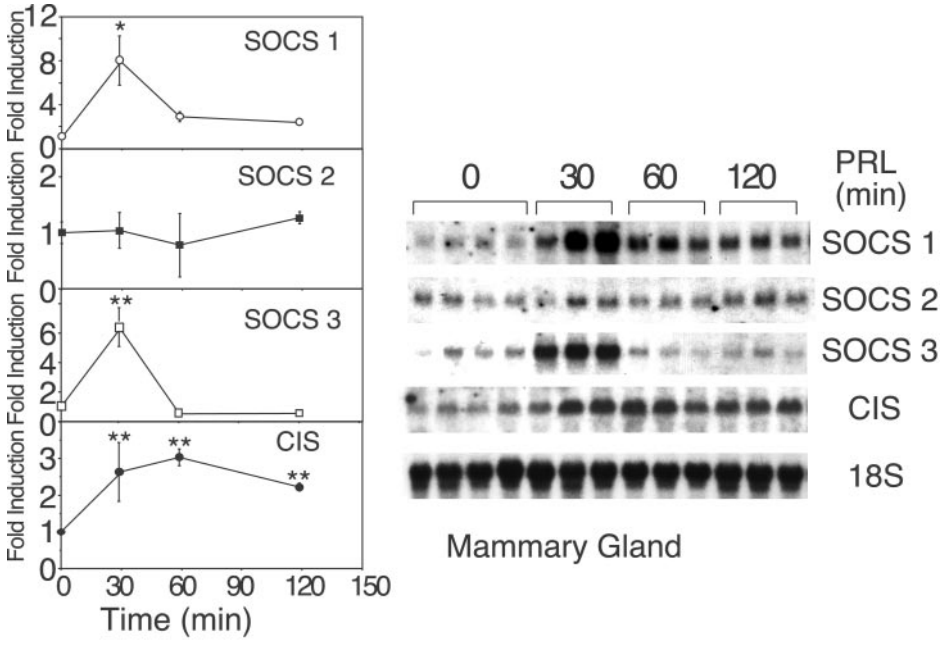

FIG. 8. Time course of induction of mRNAs for SOCS family members in response to PRL in mammary gland after 48 h of PRL and pup deprivation. Procedures are given in Fig. 1; analysis was with 20 μg of total RNA (n = 3; *, $P < 0.05$).

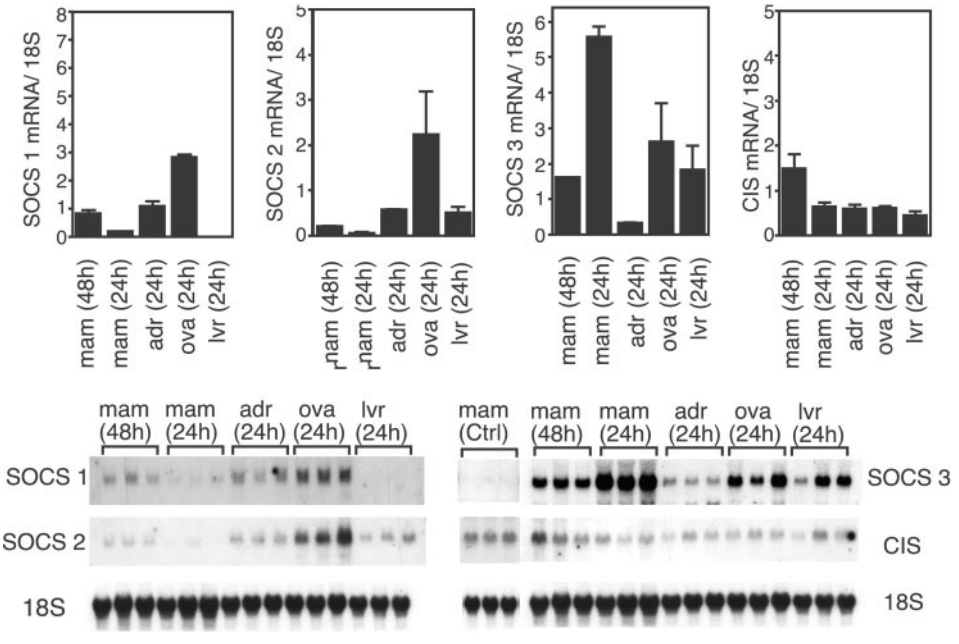

FIG. 9. Control (unstimulated) levels of SOCS mRNA in different tissues (*i.e.* in the absence of suckling or PRL administration). Total RNA was extracted from mammary gland (mam), adrenal gland (adr), ovary (ova), and liver (lvr) and analyzed by Northern blotting as described in *Materials and Methods*.

resistance in the liver (45), and for desensitization to GH-dependent induction of the insulin-like binding protein-3 acid-labile subunit after IL-1β treatment (46). In the case of the mammary gland, the cause of the increased SOCS-3 could be an increase in active STAT-3, which is known to occur within 12 h of cessation of suckling, with a concomitant decrease in active STAT 5a (47). As indicated above, the SOCS-3 gene possesses a critical STAT 3 element proximal to the transcription start site (41). Up-regulation of SOCS-3 by STAT 3 as a result of milk stasis would prevent activation and action of STAT 5a by PRL, resulting in the cessation of transcription of new milk protein mRNAs. This would provide an effective feedback loop to prevent overstimulation of milk production in the absence of suckling.

Interestingly, mammary-specific knockout of STAT 3 using the cre-lox (cre recombinance-lox P sites) approach prevented the first phase of involution, during which expression of milk protein genes is down-regulated, active STAT 5a is decreased, active STAT 3 is increased, and apoptosis genes are induced in alveolar epithelium (48). We predict that the activation of SOCS-3 would be absent or much reduced in the mammary glands of such conditional STAT 3 knockout mice and that this would be associated with a sustained sensitivity to PRL. The mechanism for the activation of STAT 3 is not known, but it could be a result of the activation of a tyrosine kinase by hydrostatic pressure within the engorged gland, possibly mediated through extracellular matrix interactions (49, 50) or the accumulation of the feedback inhibitor of

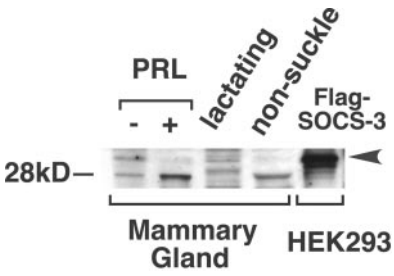

FIG. 10. SOCS-3 is induced by PRL and by pup withdrawal in mammary gland. Total cell lysate were obtained from mammary gland of 48-h bromocriptine-treated rats injected with PRL or vehicle for 60 min and from mammary gland of lactating mothers with or without pup withdrawal for 16 h. Lysates were electrophoresed on SDS-PAGE and immunoblotted with goat anti-SOCS-3 followed by HRP-conjugated antigoat antiserum and visualized by chemiluminescence. HEK293 cells transiently transfected with FLAG-tagged SOCS-3 vector were used as a positive control (*arrowhead*). The molecular mass position of endogenous SOCS-3 is shown.

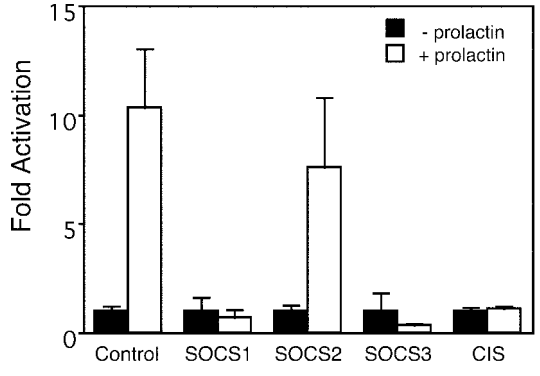

FIG. 11. Effect of coexpression of SOCS family members on the ability of PRL to transactivate the β-lactoglobulin promoter. COS-1 cells were transfected with expression vectors encoding rabbit PRL receptor, Stat 5a, a β-lactoglobulin-CAT reporter gene, and β-galactosidase. Expression plasmids encoding SOCS-1, SOCS-2, SOCS-3, and CIS were cotransfected as indicated. pUC-18 was added as required to give an equal amount of total DNA transfected. Transfected cells were either not treated (*solid bars*) or treated for 24 h with 500 ng/ml PRL (*open bars*). β-Galactosidase assays were carried out to monitor transfection efficiency. CAT activities were determined and normalized with the β-galactosidase activities. Values represent the means, and *error bars* represent SE of the triplicate data of a representative experiment. This experiment was repeated four times.

lactation, a small protein secreted with the milk and thought to act through receptors on the apical surface of the epithelial cell to inhibit lactation (51). We have found (P. Lau, S.P. Tam, and M.J. Waters, unpublished data) that a number of proteins show markedly increased tyrosine phosphorylation in 24-h pup-deprived, bromocriptine-treated mammary glands compared with those of suckling animals. These proteins are presumably targets of activated tyrosine kinase(s) that could also activate STAT 3. The identity of the responsible kinases is not yet known, but it does not include *src* or *fyn* kinases. Whatever the identity of the kinase(s), up-regulation of SOCS-3 provides a basis for the conclusion of Li *et al.* (47) that "the PRL signaling pathway is closed by local factors during the first stage of involution." Our finding that responsiveness to PRL returns 48 h after pup withdrawal, before second stage involution (lobulo-alveolar breakdown), could fit with either hypothesis, because feedback inhibition of lactation would be decreased after milk production ceases, or alternatively, opening of the tight junctions, which has occurred by this time, would decrease the milk hydrostatic pressure (52). This would allow suckling-induced systemic hormones to prevent second stage involution in teat-sealed mice, as has been observed (47).

One curious feature of our study is the observed ability of administered PRL to increase activated STAT 5a in 24-h pup-deprived mothers. This does not support the arguments described above if SOCS-3 is acting at the level of STAT 5a activation. However, we found complete inhibition of the action of STAT 5a at the promoter level in transient assays by SOCS-3 and blockade of SOCS mRNA induction *in vivo*. One could reconcile these observations by postulating a direct inhibitory action of SOCS-3 or another regulatory protein (such as p53) at the promoter level. It is known that p53 is induced within 1 day of litter removal (53, 54) and that p53 is able to inhibit transactivation by STAT 5 in reporter assays without affecting the ability of STAT 5 to bind to its response element (55). However, p53 expression remains increased for several days after pup removal (53, 54), whereas SOCS 3 declines as PRL responsiveness returns by 48 h after weaning. Currently, we are unaware of data supporting the hypothesis that SOCS 3 has a direct nuclear action, but neither are there data refuting this view, and this question is being actively investigated in our laboratory. The situation in the lactating rat evidently differs from that seen in the rabbit, in which we previously showed PRL-dependent refractoriness to PRL-induced tyrosine phosphorylation of its receptor *in vivo* (14). This refractoriness could be a result of the presence of PRL-induced SOCS, which would be appropriate in a species that suckles at more infrequent intervals than the rat. A model that accounts for the role of SOCS 3 in the regulation of mammary gland sensitivity to PRL is shown in Fig. 13.

In our reporter assay based on the β-lactoglobulin promoter, we found that CIS is able to inhibit receptor transactivation, in contrast to the reports of two other groups (43, 56), although Matsumoto *et al.* (57) reported inhibition of PRL activation of a STAT 5-based reporter by CIS that could be overcome with excess STAT 5a expression plasmid, and others have reported CIS inhibition of GH signaling inversely proportional to the level of cellular JAK 2 (58). In the latter case, inhibition by CIS required C-terminal receptor tyrosines known to bind STAT 5. It is true that transient reporter assays may not be representative of the physiological state, but in the CIS-overexpressing mouse, which expresses mammary CIS mRNA at only three to five times basal levels, there is a failure to lactate as a result of the failure of terminal end bud differentiation, and this is associated with a major decline in activated STAT 5 (57). Recently, Ram and Waxman (27) showed that CIS acts to inhibit GH signaling not by inhibiting JAK 2 [as for SOCS-1 and, to some extent, SOCS-3 (26)] or blocking access of STATs to the tyrosine-phosphorylated receptor (as for SOCS-3) but by increasing the rate of proteasomal degradation of the active hormone receptor complex over a more extended period. This interesting mechanism could reconcile the reported literature differences regarding CIS inhibition and supports our findings that CIS is capable of inhibiting STAT 5-mediated activation of a milk protein promoter. Redundancy of action between induced

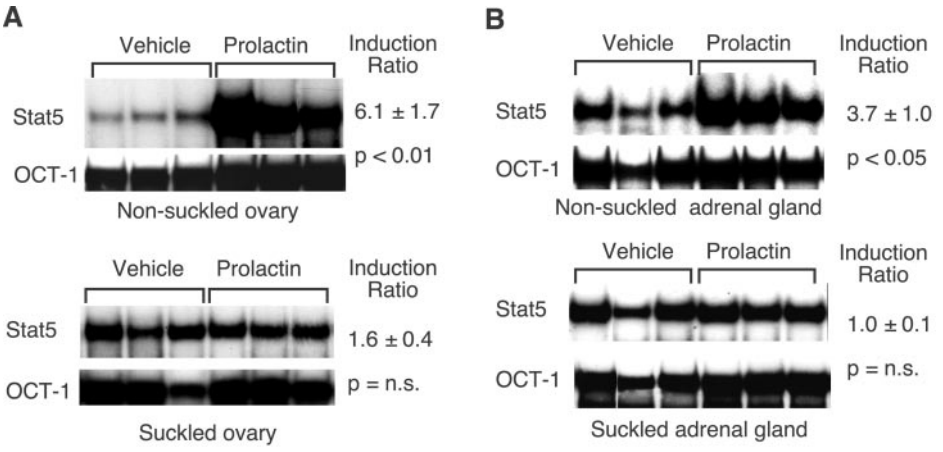

FIG. 12. Effect of a previous suckling episode on the ability of PRL to induce STAT 5 bandshift in ovary (A) and adrenal gland (B). Mothers deprived of pups for 16 h were returned to their pups for 1 h (or not, in the case of controls), administered PRL after another 1 h, and killed 30 min later. Tissues were then processed for nuclear extracts and EMSA as described in *Materials and Methods*.

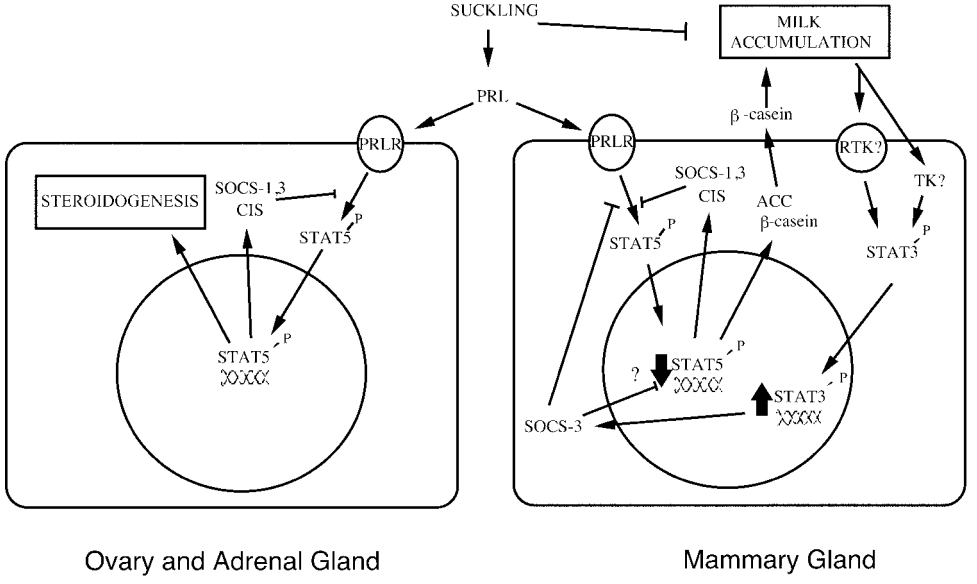

FIG. 13. Model for the interaction between PRL signaling, SOCS proteins, and filling of the mammary gland.

SOCS 1, SOCS-3, and CIS is likely to be the case physiologically.

The role of SOCS-2 remains enigmatic, and it was only induced in the ovary. Although it is induced by PRL, it does not inhibit PRL activation of the lactoglobulin promoter, and its overexpression actually increased GH transactivation of the serine protease inhibitor 2.1 gene (28). SOCS-2 does not inhibit interferon-γ-mediated antiviral and antiproliferative actions, whereas SOCS-1 and SOCS-3 do (58), and it does not inhibit leukemia inhibitory factor-mediated differentiation and growth arrest, whereas SOCS-3 does (59). It has been proposed that the delayed induction of SOCS-2 may attenuate the inhibition of signaling by SOCS-1, because this SOCS is evidently able to overcome inhibition by SOCS-1 (43). On the other hand, the SOCS-2 knockout mouse shows increased growth postnatally, apparently with increased activity of the GH axis (32). The relationship of this finding to the report that SOCS-2 binds to the IGF-1 receptor *in vivo* (60) remains to be established.

In summary, we have presented evidence that SOCS genes are induced by PRL in a tissue-specific manner and that SOCS-1, SOCS-3, and CIS, but not SOCS-2, are capable of abrogating PRL-dependent transactivation of a STAT 5-responsive gene *in vivo*. Although it appears that SOCS gene induction acts in a feedback manner to regulate the sensitivity of individual tissues to PRL secretory pulses, it is the chronic up-regulation of SOCS-3 by other means, as observed in the nonsuckling mammary gland, that may be of most physiological importance in the regulation of tissue sensitivity to PRL.

Acknowledgments

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