Nuclear targeting of stanniocalcin to mammary gland alveolar cells during pregnancy and lactation

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Hasilo, Craig P., Christopher R. McCudden, J. Ryan J. Gillespie, Kathi A. James, Edward R. Hirvi, Deenaz Zaidi, and Graham F. Wagner. Nuclear targeting of stanniocalcin to mammary gland alveolar cells during pregnancy and lactation. Am J Physiol Endocrinol Metab 289:E634–E642, 2005; doi:10.1152/ajpendo.00098.2005.—In most mammalian tissues, the stanniocalcin-1 gene (STC-1) produces a 50-kDa polypeptide hormone known as STC50. Within the ovaries, however, the STC-1 gene generates three higher-molecular-mass variants known as big STC. Big STC is targeted locally to corpus luteal cells to block progesterone release. During pregnancy and lactation, however, ovarian big STC production increases markedly, and the hormone is released into the serum. During lactation, this increase in hormone production is dependent on a suckling stimulus, suggesting that ovarian big STC may have regulatory effects on the lactating mammary gland. In this report, we have addressed this possibility. Our results revealed that virgin mammary tissue contained large numbers of membrane- and mitochondrial-associated STC receptors. However, as pregnancy progressed into lactation, there was a decline in receptor densities on both organelles and a corresponding rise in nuclear receptor density, most of which were on milk-producing, alveolar cells. This was accompanied by nuclear sequestration of the ligand. Sequestered STC resolved as one ~135-kDa band in the native state and therefore had the appearance of a big STC variant. However, chemical reduction collapsed this one band into six closely spaced, lower-molecular-mass species (28–41 kDa). Mammary gland STC production also underwent a dramatic shift during pregnancy and lactation. High levels of STC gene expression were observed in mammary tissue from virgin and pregnant rats. However, gene expression then fell to nearly undetectable levels during lactation, coinciding with the rise in nuclear targeting. These findings have thus shown that the mammary glands are indeed targeted by STC, even in the virgin state. They have further shown that there are marked changes in this targeting pathway during pregnancy and lactation, accompanied by a switch in ligand source (endogenous to exogenous). They also represent the first example of nuclear targeting by STC.

nucleus; mitochondria; membrane; receptor

IN MANY MAMMALIAN NEONATES SUCH AS RODENTS, the maternal contribution includes two factors mandatory to survival. Colostrum aids in providing the neonate with passive immunity against infection, whereas milk contains the appropriate nutritional requirements for sustaining postnatal growth. Both are provided by the mammary glands. Mammary tissue also has high calcium requirements during lactation, and there is good evidence that calcitropic hormones such as parathyroid hormone-related protein have important roles in their development and during lactation (19). Stanniocalcin (STC) is another calcitropic hormone that initially evolved as a means of regulating extracellular calcium balance and that may also play a role in mammary gland function.

STC was initially characterized as a 50-kDa, homodimeric polypeptide in fish (39) and was subsequently discovered in mammals. Both human and mouse cDNAs encode proteins of 247 residues that share 73% overall sequence homology to fish STC (3, 4, 29). Furthermore, as in fishes, mammalian STC was initially discovered to be a 50-kDa homodimer. The mammalian STC gene is widely expressed in humans and rodents (3, 4, 38), but in contrast to fish the mammalian hormone is virtually undetectable in serum (6). This appears to be due to its rapid sequestration by erythrocytes, which contain large numbers of high-affinity binding sites (18). High serum hormone levels have been achieved, however, in transgenic over-expressing mice. In transgenic lines where the transgene is widely expressed (heart, liver, and brain), the phenotypes of medium- and high-expressing lines include intrauterine and postnatal developmental delay (dwarfism) and significant reductions in litter size (37). However, lower levels of transgene expression that are confined solely to skeletal muscle cells produce an energy-wasting phenotype, hyperphagia, and enhanced respiration (10). There was also mitochondrial swelling in muscle cells (10), likely due to excessive STC targeting (26).

In the mouse, STC is readily detectable in the serum only during pregnancy and lactation. The hormone first becomes detectable in the serum on day 6 of pregnancy, peaks on day 14, and then remains elevated throughout the lactation phase. coincide with the rise in serum STC levels, ovarian STC gene expression rises as much as 15-fold and also remains elevated during the lactation phase (7). Indeed, it is because the profiles of serum STC and ovarian STC expression are so closely correlated that the ovaries are thought to be the source of the blood-borne hormone.

The ability of ovary-derived STC to circulate during pregnancy and lactation likely derives from its unique structure. Unlike the 50-kDa form of STC present in most tissues, ovarian STC consists of three higher-molecular-mass species of 84, 112, and 135 kDa that are collectively known as big STC (31). These ovarian forms of STC are normally targeted locally to luteal cells (30). However, they may be targeted elsewhere during pregnancy and lactation. The lactating mammary gland could be one such target, based on the fact that ovarian STC gene expression is so highly dependent on the suckling stim-

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were obtained from Dr. A. F. Parlow of the NIH-NHPP. Histological analysis was performed on three animals per group. D14P and on D4PW and from three animals on D7L and D18L. Glands. For the subcellular determination of receptor and protein Rats were not fasted before they were killed. Weaning was initiated lactation (D7L and D18L), and the 4th day postweaning (D4PW). Virgin rats were a minimum of 8 wk of age. Pregnant and lactating 14th days of pregnancy (D12P and D14P), the 7th and 18th days of ob- tained for stage-specific histological and biochemical analysis of mammary gland STC receptors, STC ligand, and STC mRNA levels. MATERIALS AND METHODS Experimental animals. Virgin, pregnant, and lactating female Wistar rats (Charles River Laboratories, Montreal, QC, Canada) were obtained for stage-specific histological and biochemical analysis of mammary gland STC receptors, STC ligand, and STC mRNA levels. Virgin rats were a minimum of 8 wk of age. Pregnant and lactating rats were a minimum of 12 wk of age. Rats were killed on the 12th and 14th days of pregnancy (D12P and D14P), the 7th and 18th days of lactation (D7L and D18L), and the 4th day postweaning (D4PW). Rats were not fasted before they were killed. Weaning was initiated after 21 days of nursing by removal of the pups. All analyses were performed on bilaterally dissected abdominal and inguinal mammary glands. For the subcellular determination of receptor and protein levels, mammary glands were pooled from four animals on D12P and D14P and on D4PW and from three animals on D7L and D18L. Histological analysis was performed on three animals per group. Reagents. Ovine LH (oLH), ovine FSH (oFSH), and rat prolactin (pPRL) were obtained from Dr. A. F. Parlow of the NIH-NHPP (Harbor-UCLA Medical Center, Torrance, CA). Human STC-related protein (hSTC1P or hSTC2) and recombinant human STC (rhSTC) were obtained from Human Genome Science (Rockville, MD). A fusion protein of rhSTC and alkaline phosphatase (STC·AP) was generated in MDCK cells for use as a ligand in the receptor-binding assays and for in situ ligand binding. Human AP was generated in the same cell line (no STC insert) and employed as a control in both receptor assays (26).

Histological localization of STC ligand and receptor. Rats were anesthetized with an intraperitoneal injection of Somnitol (63 mg/kg) and perfused intracardially with phosphate-buffered saline (PBS), pH 7.4, followed by PBS containing 4% paraformaldehyde (PFA). Mammary glands were then removed, postfixed overnight in PFA, and embedded in paraffin. Tissue sections were cut to a thickness of 6 μm. In situ ligand binding was performed as previously described for the cellular localization of STC receptors (26, 30). The method employs a fusion protein of STC and human placental AP, known as STC·AP, stably expressed in MDCK cells (26). Briefly, tissue sections were equilibrated in Hanks’ balanced salt solution containing 0.1% BSA, pH 7.5 (HBHA buffer), and then incubated for 90 min in HBHA buffer containing 1 nM STC·AP. Control slides were incubated in either AP alone or STC·AP containing 1 μM rhSTC. Slides were then washed and processed for visualization of bound AP activity, as described (26). Three animals were analyzed in each of the virgin, pregnant, lactating, and postweaning groups. Immunochemistry (ICC) was performed as previously described, using an rhSTC antiserum generated in rabbits against bac- ulovirus-expressed rhSTC. The antiserum has been previously characterized for specificity by ICC and Western blot analysis (6, 7). Briefly, tissue sections were incubated overnight at 4°C with a 1:1,000 dilution of rabbit anti-rhSTC serum. The sites of antibody binding were visualized with biotinylated secondary antibodies and the Vectastain ABC peroxidase detection system (Vector Laboratories, Burlington, CA) or with FITC-conjugated goat anti-rabbit gamma globulin. As staining controls, tissue sections were incubated in normal rabbit serum (NRS) in lieu of antiserum. Three animals were analyzed per group in this manner (virgin, pregnant, lactating, and postweaning). Images were captured on a Nikon E1000 upright bright-field microscope (Nikon Canada, Mississauga, ON, Canada) with a DCM1200 digital camera and ACT-1 software (Nikon Canada).

To verify STC ligand and receptor staining of cell nuclei, slides were counterstained with 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) dissolved in PBS. In the case of STC ligand localization by fluorescent ICC, the same slides were counterstained with DAPI. In the case of STC receptor localization with STC·AP, adjacent sections were stained with DAPI. In both cases, tissue sections were stained for 10 min in DAPI solution, washed in PBS, and mounted for confocal imaging (Bio-Rad Radiance 2000 laser scanning system).

Subcellular fraction of rat mammary glands. Cell fractions were prepared as previously described (12). Briefly, pooled tissues were minced with scissors and then homogenized in 5 volumes of 0.25 M sucrose containing 0.005 M MgCl₂ and 0.010 M Tris·HCl, pH 7.4, using a motor-driven Potter-Elvehjem homogenizer with a loose-fitting Teflon pestle. The homogenate was then centrifuged twice at 1,000 g for 15 min, once at 3,000 g for 15 min, and finally at 100,000 g for 90 min, thereby isolating crude nuclear, mitochondrial, and microsomal membrane fractions, respectively. Higher-purity nuclear fractions were obtained by density gradient centrifugation of the 1,000-g crude nuclear fraction (13, 14). Briefly, the pellet obtained from the 1,000-g spin was resuspended in 1 ml of 0.25 M sucrose homogenization medium, followed by homogenization in 9 volumes of 2 M sucrose containing 1 mM MgCl₂ and 10 mM Tris·HCl, pH 7.4. The resulting suspension was layered on a 2-ml cushion of 2.2 M sucrose and overlaid with 1 ml of 1 M sucrose. The sample was then centrifuged at 100,000 g for 90 min at 4°C. All isolated subcellular fractions were quantified for total protein content using the Bio-Rad Protein Assay Kit and stored at −70°C for subsequent analysis in receptor-binding assays. Transmission electron microscopy was used to verify the integration of purified cell nuclei. Nuclear pellets were fixed in a mixture of 2% glutaraldehyde-2% PFA in 0.1 M phosphate buffer, pH 7.4, for 3 h and embedded in Lowicryl K4M. Ultrathin sections were stained with 1% uranyl acetate for 30 s (Marivac, Halifax, NS, Canada) and analyzed with a Phillips EM300 transmission electron microscope operating at 60 kV.

Receptor-binding assays. Saturation binding assays were carried out on the purified mammary gland nucleus and microsomal membranes pooled from three or four animals to obtain estimates of Kₐ and Bₘₐₓ. Mitochondria were pooled from two animals at each stage. Aliquots of each fraction (25–200 μg/tube) were incubated in triplicate with increasing amounts of STC·AP or the equivalent amount of AP activity as previously described (26). For nuclei, 200 μg of nuclear protein were used per tube compared with 100 μg/tube for membranes and 50 μg/tube for mitochondria. Specific binding was obtained by subtracting AP binding from total STC·AP binding. The specificity of STC·AP binding was assessed on 200-μg aliquots of microsomal membranes from D18L mammary gland. Membranes were incubated with fixed amounts of STC·AP (500 nM) ± 1.0 μM oLH, oFSH, pPRL, hSTC1P (hSTC2), rhSTC, or STC·AP alone for 90 min at room temperature (5 replicates per hormone). Further aliquots of membrane were incubated with equivalent amounts of AP alone as a control. The separation of bound and free ligand and the subsequent detection of AP activity were performed as described above.

Western blot analysis. Proteins were extracted from purified nuclei by use of a low pH treatment, as previously described (12, 15). Isolated mammary gland nuclei were resuspended in 0.1 M glycine, pH 3.0, containing 0.1% Triton X and incubated overnight at 4°C to elute bound nuclear proteins. Nuclear proteins were concentrated with Centricon-10 microconcentrators (Millipore, Bedford, MD), being buffer exchanged with 0.05 M Tris·HCl, pH 7.0, and resolved on SDS-PAGE gels under reducing (0.5% β-mercaptoethanol) and non- reducing conditions. rhSTC50 was also run for size comparison. Western blot analysis was performed as previously described, using a 1:40,000 dilution of rhSTC antiserum (26, 30). Blots were subse- quently developed with an ECL Western blotting detection kit (Am- ersham Pharmacia Biotech, Baie d’Urfe, QC, Canada). Control blots were incubated with antiserum preabsorbed with 1 μg/ml STC to identify all STC-specific bands.

Radioimmunoassay. The STC content of serum samples was quanti- fied by radioimmunoassay (RIA). Serum samples were analyzed in
triplicate on 100-μl aliquots of serum (5 animals/group). The RIA has been characterized for specificity and has a detection limit of 0.2 ng/ml (6).

Northern blot analysis. STC gene expression was monitored in mammary glands from virgin, pregnant, lactating, and postweaning females. Abdominal and inguinal mammary glands were removed and pooled from five animals per group for RNA isolation. Total RNA was isolated using TRIzol according to the manufacturer’s instructions (Life Technologies, Burlington, ON, Canada). Northern blot analysis was performed on 30-μg aliquots of RNA from each group as previously described (31). Blots were prehybridized in Ambion ULTRAhyb (Ambion, Austin, TX) for 2 h at 42°C and probed overnight with a random primed, 32P-labeled, 815-bp mouse STC cDNA fragment (2 × 106 cpm/ml) in ULTRAhyb at 42°C. Blots were rehybridized with a 32P-labeled 18S RNA probe to normalize for loading. Densitometric analysis was performed to determine STC-to-18S RNA ratios by use of Image Master VDS Quantification Software version 2.0 (Amersham Pharmacia Biotech).

GraphPad Prism (GraphPad Software, San Diego, CA) using a non-linear regression analysis of one-site binding to obtain estimates of Kd and Bmax. Comparisons of specific binding between groups in virgin, pregnant, lactating, and postweaning animals were performed with a one-way ANOVA followed by Dunnett’s test, with virgins serving as the control group. Comparisons of serum STC levels between groups were performed with a one-way ANOVA followed by a Student-Newman-Keuls test. Statistical significance was assumed if P < 0.05.

RESULTS

Histological localization of STC receptors by in situ ligand binding. In situ ligand binding (ISLB) studies revealed discrete, displaceable binding of STC·AP to cells in specific regions of the mammary gland. Moreover, the distribution of binding sites varied at different stages of development and involution. Figure 1A is a sagittal view of a virgin rat mammary gland lactiferous duct. The purple and lighter-brown staining is indicative of specific ligand binding in greater and lesser amounts, respectively. The highest levels of ligand binding were observed in ducetal epithelial cells (e; arrowhead) lining the basement membrane. At higher magnification (inset, top right) the staining was purple and punctate in appearance and highly reminiscent of the mitochondrial receptor staining seen in rat liver and kidney (26). The same punctate staining pattern was also evident in adipocytes (a; arrowhead) of the mammary gland stromal compartment. Lower levels of binding were present more apically in the cytoplasm of ductal epithelial cells (asterisk in inset) and in the connective tissue layer (c) surrounding the duct. Tissue sections incubated with STC·AP plus rhSTC50 or AP alone exhibited no staining (inset, bottom right).

An entirely different pattern of receptor localization was observed in the developing mammary gland, commencing on D14P as shown in Fig. 1B. Concomitant with lobuloalveolar development, intense ligand binding was observed over the nuclei of alveolar cells (blue arrows). Less pronounced staining was evident over the cell cytoplasm (black arrow in inset, top right). Ligand binding to adipocytes was still evident but less so than in virgins. No staining was observed in control slides (inset, bottom right).

Ligand binding to alveolar cells became even more pronounced during the nursing period. On D7L, intense binding was now evident over many of the cell nuclei in individual alveoli (Fig. 1C, blue arrows). These structures were identified as nuclei by DAPI staining of adjacent tissue sections (not shown). Intense binding was also evident over regions corresponding to the basolateral alveolar cell membrane adjacent to the capillaries (black arrowheads). The basolateral membrane staining was clearly more concentrated in specific areas (inset, top right). No staining was obtained in control slides treated with STC·AP plus rhSTC (Fig. 1C, bottom right inset). The punctate pattern of receptor localization in adipocytes was no longer evident by D7L, likely on account of the decline in adipocyte number (results not shown).

A similar pattern of basolateral membrane and nuclear staining was evident in mammary gland on D18L, although basolateral membrane staining was not as evident (results not shown). By D4PW, however, significant changes had occurred in the now-involuting mammary gland. Nuclear staining was no longer present, and the punctate pattern of receptor localization seen in the virgin mammary gland was beginning to become evident once more. On the whole, ligand binding was unremarkable in the involuting mammary gland, purely cytoplasmic in its distribution pattern within the cells and equally distributed in alveolar cells, adipocytes, and cells within the connective tissue layer (results not shown).

ICC. In virgin rats, the highest levels of STC immunoreactivity (STCir) were observed in fibroblasts and adipocytes of the mammary gland stroma (results not shown). Coincident with the change in pattern of ISLB staining on D14P, the pattern of STCir also changed. Diffuse cytoplasmic staining was evident in alveolar cells. With the onset of lactation, the ligand now began to exhibit a nuclear localization pattern in alveolar cells, but with one notable difference (Fig. 1, D–F). Whereas ISLB staining appeared to be homogeneously distributed throughout the nucleoplasm (Fig. 1C, blue arrows), antibody staining for the ligand was generally concentrated on the nuclear envelope, as evidenced by its colocalization with DAPI (Fig. 1, D–F, yellow arrows). Some nuclei showed little or no staining (Fig. 1, D–F, white arrows). Antibody staining was also evident on the membranes of lipid droplets as they formed and budded from apical membranes (Fig. 1D, small yellow arrowheads) and within the milk proper (Fig. 1D, large yellow arrowheads).

By D4PW, only low levels of STCir were evident in the mammary gland. Basolateral membrane immunostaining was no longer evident, and low levels of STCir were infrequently associated with the nuclei of regressing alveolar cells. STCir was also evident in the milk droplets of involuting lobules (not shown). In addition, weak staining was present in the alveolar cells of regressing lobules (results not shown).

In control tissue sections, there was no immunostaining when the antiserum was blocked with STC (Fig. 1D, bottom left inset).

Mammary gland receptor-binding studies. The changes in mammary gland receptor localization, as revealed by ISLB, prompted us to characterize the receptor-binding activity in isolated nuclear, microsomal membrane, and mitochondrial fractions. To first of all address the specificity of STC·AP binding to mammary gland receptors, oLH, oFSH, rPRL, and STC2 (STCrP) were tested for their relative abilities to displace ligand binding to microsomal membrane STC receptors. The results showed that rhSTC and, to a lesser extent, STC2 were able to significantly displace STC·AP binding at concentrations of 1.0 μM (Fig. 2).
Fig. 1. Histological localization of stanniocalcin (STC) ligand and receptor in rat mammary gland. A: STC receptors in virgin mammary gland showing a sagittal view of a lactiferous duct (×600). Intense binding of STC-alkaline phosphatase (STC-AP) to ductal epithelial cells (e and white arrow, top right inset) and stromal cells at their interface along the basement membrane (c) produces the purple punctate pattern of staining for receptors. This same punctate staining pattern was also present in adipocytes (a) of the stromal compartment. Tissue sections incubated with STC-AP + recombinant human (h)STC or AP alone (bottom right inset) exhibited no staining. L, ductal lumen. B: STC receptors in day 14 pregnant (D14P) mammary gland (×600). STC-AP binding shows the presence of large nos. of STC receptors in alveolar cell nuclei (blue arrows). Note abundance of darkly stained nuclei. A weaker, reddish-brown cytoplasmic stain is also evident in alveolar cells (black arrow, top right inset). Punctate staining pattern evident over adipocytes in A is now absent. No specific binding was evident in control AP-treated sections (bottom right inset). C: STC receptors in day 7 lactating (D7L) mammary gland (×600). Intense binding is evident over most alveolar cell nuclei (blue arrows) and to regions of basolateral membrane adjacent to the capillaries (black arrowheads). Basolateral membrane staining was more concentrated in specific areas (top right inset). No staining was obtained in AP-treated control slides (bottom right inset). L, alveolar lumen. D: STC protein in D7L mammary gland as revealed by confocal fluorescent immunocytochemistry (ICC; 1:1,000 antiserum dilution). STC immunoreactivity is evident over much of the cell cytoplasm. More intense immunoreactivity is apparent on some nuclear membranes (yellow arrows) or throughout select nuclei (pink arrow); see E and F for comparison. Other nuclei are notable by their relative lack of STC immunoreactivity (white arrows). STC is also evident on membranes of lipid droplets within the alveolar lumen (big yellow arrowhead) and apically on alveolar cells during lipid droplet formation (small yellow arrowhead). Top right inset: control section treated with antibody + rhSTC shows no staining. L, alveolar lumen. E: DAPI staining of cell nuclei (blue) in tissue section shown in D. Arrows and arrowheads correspond to those in D and F. F: overlay of D and E reveals nuclear-associated STC as a lighter shade of blue. Yellow arrows highlight nuclei with STC confined to the nuclear envelope (yellow arrows). Pink arrow highlights a nucleus that is stained throughout. White arrows highlight nuclei with little or no STC immunoreactivity. Small and large yellow arrowheads denote STC-immunoreactive lipid droplets during and after their formation, respectively. L, alveolar lumen. Bottom right inset: STC ligand concentrated on the nuclear envelope of an alveolar cell (arrow) as revealed by ICC using the peroxidase detection system. STC immunoreactivity is also evident on nearby basolateral membranes (arrowhead). Scale bars, 50 μm.
Membrane receptors. Having established the specificity of STC-AP binding, we proceeded to examine the binding properties of isolated membranes, nuclei, and mitochondria. The analysis of membrane receptor levels from virgin, pregnant, lactating, and postweaning mammary glands is shown in Fig. 3 and summarized in Table 1. Values of $B_{\text{max}}$ rose from 17.4 ± 1.0 pmol/mg protein (means ± SE) in virginis to a high of 19.4 ± 1.5 pmol/mg protein in D14P mammary gland. The values of $B_{\text{max}}$ fell thereafter during lactation and postweaning to 12.2 ± 1.7 pmol/mg protein on D7L ($P < 0.05$, ANOVA and Dunnett’s test), 9.7 ± 2.0 pmol/mg protein on D18L ($P < 0.01$), and 7.8 ± 0.9 pmol/mg protein on D4PW ($P < 0.01$; Table 1). Estimates of affinity yielded a $K_d$ of 0.8 ± 0.1 nM for virgin receptors, 0.3 ± 0.1 nM on D14P, 1.0 ± 0.4 nM on D7L, 2.5 ± 1.1 nM on D18L, and 1.8 ± 0.5 nM on D4PW. These values of $K_d$ fell within a similar range to that obtained for the nuclear receptors (Table 1).

Nuclear receptors. The results obtained with nuclei from pregnant and lactating mothers are shown in Fig. 4 and summarized in Table 1. Here, it can be seen that saturable, high-affinity binding sites were obtained in all cases. Estimates of $K_d$ were not significantly different at all four time points, ranging from 1.4 ± 0.6 nM on D12P, 1.96 ± 0.7 nM on D14P, and 0.75 ± 0.2 nM on D7L to 1.7 ± 0.6 nM on D18L. In contrast, the nuclear binding capacity for STC ($B_{\text{max}}$) increased in stepwise fashion approximately threefold over pregnancy and lactation (Fig. 4). The values of $B_{\text{max}}$ increased from 3.4 ± 0.6 pmol/mg protein on D12P to 5.6 ± 1.0 pmol/mg protein on D14P. Then, from pregnancy to lactation, $B_{\text{max}}$ rose to 7.9 ± 0.5 pmol/mg protein on D7L ($P < 0.01$) to 9.6 ± 1.6 pmol/mg protein on D18L (Table 1; $P < 0.01$, ANOVA and Dunnett’s test). Therefore, $B_{\text{max}}$ increased progressively over pregnancy and lactation, whereas $K_d$ remained essentially unchanged. The yields of nuclei from virgin and postweaning mammary tissue were low by comparison and so precluded the same type of analysis.

Mitochondrial receptors. Binding studies on isolated mitochondria revealed a changing pattern of $B_{\text{max}}$ that was similar to that observed in membranes. As reflected in the ISLB studies (Fig. 1A), mitochondrial targeting was significantly higher in virgins than at all other time points (Fig. 5 and Table 1; $P < 0.01$, ANOVA and Dunnett’s test). When the data were normalized for protein content, there proved to be fivefold more receptors in virgin mitochondria ($B_{\text{max}}$ of 169.70 ± 25.0 pmol/mg protein) than during lactation (32.8 ± 3.5 pmol/mg protein on D7L and 32.4 ± 7.3 pmol/mg protein on D18L) and the postweaning period (29.4 ± 2.1 pmol/mg protein). The drop in mitochondrial receptor number was also accompanied by a change in receptor affinity. In virgins, the receptor had an estimated $K_d$ of 12.4 ± 2.7 nM. Coinciding with the decline in receptor number, there was a statistically significant decline in $K_d$ (5-fold increase in affinity), such that the estimated $K_d$ for D7L mitochondrial receptors was 1.5 ± 0.4 nM, 2.7 ± 1.5 nM on D18L, and 2.7 ± 0.5 nM on D4PW (Fig. 5 and Table 1; $P < 0.01$ ANOVA and Dunnett’s test).

Circulating STC levels during pregnancy and lactation. The profile of serum STC levels in the rat was somewhat similar to that reported in pregnant and lactating mice (7). As shown in Fig. 6, serum STC levels rose from 0.24 ± 0.02 ng/ml in virgins ($n = 5$) to a high of 0.35 ± 0.04 ng/ml on D12P ($P < 0.01$; ANOVA and Dunnett’s test). Hormone levels declined thereafter to 0.31 ± 0.04 ng/ml on D14P and even further to 0.27 ± 0.61 ng/ml on D7L. Serum STC levels were below RIA detection limits on D18L and during the postweaning period.

Characterization of nuclear STC. In light of the fact that STC immunoreactivity was visible in the nucleus during lactation (Fig. 1, D–F), the nature of nuclear STC was assessed by Western blot analysis. Acid-extracted nuclear proteins from D7L and D18L were subjected to SDS-PAGE and Western blot analysis under both nonreducing and reducing conditions. Similar results were obtained with nuclei from D7L and D18L. Under nonreducing conditions, a single band of ~125 kDa was evident in D7L and D18L nuclear protein extracts (Fig. 7). After chemical reduction with β-mercaptoethanol, however, this one band collapsed into six lower-molecular-mass species of 28.3, 31, 33.4, 36, 38.7, and 40.9 kDa. The size difference between the reduced bands averaged 2.5 kDa (Fig. 7). To ensure the specificity of antibody staining, duplicate blots were

Fig. 3. Changes in mammary gland membrane receptor levels. Membrane receptor levels were highest in virgin and D14P females. They then fell progressively during the lactation phase and reached a low of 7.85 ± 0.89 pmol/mg protein ($B_{\text{max}}$) on day 4 postweaning (D4PW). $K_d$ values during this time were relatively stable. As described in materials and methods, microsomal membranes from virgin, D14P, D7L, D18L, and D4PW rat mammary glands were incubated with increasing amounts of STC-AP or AP, and estimates of $K_d$ and $B_{\text{max}}$ were thus determined. Each data point represents the mean ± SE of 3 replicates. Error bars <0.01 pmol are not visible.

Fig. 2. Specificity of STC-AP binding to mammary gland receptors. Purified mammary gland membrane preparations were pooled from 3 D18L animals and incubated with 500 nM STC-AP in the presence of 1.0 μM ovine (o)LH, oFSH, rat prolactin (rPRL), hSTC-related protein (STCrP or hSTC2), hSTC, or STC-AP alone. Specific binding was defined as described in materials and methods. Analysis of data showed that only STC and, to a lesser extent, STCrP were able to significantly compete with STC-AP for membrane binding sites. Data were analyzed by one-way ANOVA, and individual groups were compared by Dunnett’s test. Bars represent means ± SE of 5 replicates/group. *Significantly different from STC-AP control groups, $P > 0.05$. 
treated with the same primary antibody dilution (1:40,000) preabsorbed overnight with excess rhSTC (500 μg/ml). This control procedure abolished the nonreduced 125-kDa band and the six smaller reduced bands, suggesting that they were all STC specific (results not shown).

**Mammary gland STC gene expression.** Northern blot analysis of mammary gland total RNA was carried out to determine the pattern of STC gene expression in animals at various stages of pregnancy. The results showed that STC gene expression was readily detectable in both virgin and pregnant animals. The main transcript was 4 kb, with lesser amounts of a 2-kb transcript also in evidence (Fig. 8A). Densitometry analysis of STC-to-18S ratios revealed that STC receptor levels increased in virgin and D14P, however, receptor affinity rose. In virgins, receptor affinity was barely detectable during lactation and the postweaning phase (Fig. 8B).

**DISCUSSION**

The hypothesis that a blood-borne form of STC targets the mammary glands during pregnancy and lactation appears to have been validated by the findings in the present study. The results have shown that the STC gene is highly expressed in the virgin rat mammary gland together with high levels of membrane and mitochondrial STC receptors. However, after the onset of pregnancy and during the lactation phase, there was a progressive decline in the number of microsomal and mitochondrial receptors and a concomitant rise in nuclear receptors, specifically on milk-producing alveolar cells. The upregulation in nuclear receptors was most apparent during the lactation phase. Of equal importance in the context of our original hypothesis, as nuclear targeting was on the rise there was a concomitant decline in mammary gland STC production to nearly undetectable levels. This suggested that there was a switch in hormone source from an endogenous one to one that was almost exclusively exogenous and, therefore, systemic. Moreover, the ligand sequestered by the nucleus fell within the size range of the ovarian big STC variants (84, 112, and 135 kDa) and could very well have originated in the ovaries. However, this remains to be proved.

This study represents the first detailed analysis of STC targeting in the rodent mammary gland. Until now, studies on mammary gland have been focused for the most part on the possible involvement of STC in the pathology of breast cancer. For instance, Northern blot analysis has revealed that the STC gene is only weakly expressed in normal human breast tissue but highly expressed in invasive and ductal carcinomas, where the transcript is confined to malignant cells (2, 27). The cells responsible for STC gene expression in normal human mammary tissue have not yet been identified, but ISLB studies have revealed that STC receptors are confined mainly to stromal and ductal epithelial cells (27). The present study suggests that the same cell types are targeted in rodents, as...
ISLB staining revealed that the majority of STC receptors in virgin rat mammary gland were on ductal epithelial cells and adipocytes of the underlying stroma. Moreover, the punctate distribution pattern of receptors over these cells was highly reminiscent of that reported in liver and kidney, where STC50 is targeted to and sequestered by the mitochondrial matrix (26). The decline in mitochondrial receptors during lactation is likely due to the declining size and activity of the mammary gland stroma compartment, which consists primarily of adipocytes and undifferentiated mesenchymal cells. The glands are by this time fully developed, and the adipocytes are almost entirely fat depleted (16, 17), resulting in a stroma that is now, in relative terms, metabolically quiescent. Under these circumstances, it would appear that the mitochondria have reduced requirements for STC targeting. On the other hand, receptor affinity was increased in direct proportion to the decline in Bmax and declining mammary gland STC gene expression. This may be a means by which the mitochondria maintain an adequate level of STC receptivity in the face of falling endogenous ligand production. If true, this would suggest that mitochondrial targeting by STC continues to be of importance during lactation.

Receptor-binding studies revealed that this was indeed the case, as quantifiable mitochondrial binding sites were identified at all stages of development. Comparatively, virgin mammary gland targeting of STC
mary gland had the most mitochondrial receptors. The levels then dropped by 80% with the onset of lactation and the rise in nuclear targeting and change in ligand source (endogenous to exogenous). Another factor in this transition may have been the marked reduction that occurs in the size of the stromal cell compartment during lobuloalveolar development. The relevance of this lies in the fact that, in other developing tissues (bone, kidney, and testis), the STC gene is expressed exclusively by mesenchymal stromal cells for targeting to cells undergoing growth and differentiation, such as chondrocytes and epithelial cells (34–36). More recent data has shown that adipocytes can also be a source of STC gene expression (32). If this were also the case in mammary gland, then a contracting stromal cell compartment might naturally reduce STC production.

Along with the profound changes in gene expression and mammary gland tissue architecture that occur over pregnancy and lactation (8, 9, 16, 17, 33), the present study has documented the emergence of an entirely new STC targeting pathway. Commencing on D12P, high-affinity STC receptors became apparent on the nuclei of alveolar cells. Nuclear receptor density rose progressively such that, in lactating mammary gland, ISLB analysis revealed intense binding activity throughout the nucleoplasm in many alveolar cells. At the same time, there was a progressive decline in microsomal membrane-associated receptors. In view of the fact that both membrane and nuclear receptors had similar values of $K_d$, these findings argue in favor of an inward receptor trafficking pathway, from the cell surface to the nucleus. Histological evidence further suggests that the receptor is accompanied by the ligand. In many instances, ICC staining revealed STC ligand that colocalized with DAPI on the nuclear envelope. Hence, the possibility that the ligand-receptor complex remains on the nuclear envelope and activates a signaling cascade has to be given due consideration. In addition, some of the ligand may gain entry to the nucleus, as preliminary studies have revealed measurable levels of immunoassayable STC in crude chromatin fractions (unpublished observations). Collectively, the findings place big STC on an ever-growing class of ligands that internalize with (unpublished observations). Collectively, the findings place big STC on an ever-growing class of ligands that internalize with

The nature of any associations with chromatin and/or other nuclear proteins should reveal much in terms of STC function as nursing represents a phase of immense maternal adaptation, during which time the mammary gland is at its highest state of metabolic activity (1). By D7L, when nuclear sequestration of the hormone is readily apparent, rat pups are already growing at a rapid rate, and the demand for milk production only increases thereafter (11). In the fully developed lobules, capillaries interfacing directly with the basement membrane adjacent to the basolateral membranes of milk-producing alveolar cells allow for the continuous and rapid transfer of materials from maternal blood (25). Here, STC may activate preexisting transporters for essential milk components, such as calcium and phosphate, as it does in kidney and gut (23, 40) and at the same time increase their rates of synthesis via the nuclear targeting pathway.

Western blot analysis of the nuclear ligand revealed that it fell within the size range of the big STC variants previously described in ovary (30, 31). Little is known regarding the structure of these variants. Whereas STC50 is a dimer of identical 25-kDa subunits, big STC comprises three larger proteins of 84, 112, and 135 kDa that are thought to be progressively larger complexes (homodimers, -trimers and -tetramers) of a larger, 45-kDa subunit (30, 31). Posttranslational modifications and added protein sequence could both account for the increase in overall subunit mass from 25 to 45 kDa. These subunits obviously have in common an unspecified length of core sequence, as both cross-react with antibodies to STC50 and show parallelism in immunoassays (31), indicating that they share common antigenic determinants. In view of what is known, therefore, it was intriguing to discover that the nuclear form of STC was not reduced to a 45-kDa species following exposure to β-mercaptoethanol but instead collapsed into six molecular mass species of 28.3, 31, 33.4, 36, 38.7, and 40.9 kDa, all of which proved to be STC related on the basis of the Western blotting controls. Because their size differences fell within such a narrow range (2.2–2.7 kDa), they could be differentially phosphorylated isoforms, especially as Jellinek et al. (20) have found that STC is capable of being phosphorylated. Alternatively, they could also arise by progressive NH$_2$- or COOH-terminal truncations of the core sequence. At the very least, the data suggests that nuclear-targeted STC is a disulfide-linked complex of at least six smaller proteins that must arise as a result of extensive posttranslational processing.

In summary, we have described for the first time a targeting pathway that involves STC targeting to mammary gland alveolar cell nuclei during pregnancy and lactation. Because the pathway becomes fully operative at a time when the mammary gland all but ceases to produce STC, the targeted hormone appears to be systemically derived. The evidence continues to point to the ovaries as being the most likely source of blood-borne STC, yet this remains to be proven. Collectively, the results also provide a reasonable explanation as to why ovarian STC gene expression is upregulated during lactation and so highly dependent on the suckling stimulus.

GRANTS

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REFERENCES
