Mammary gland copper transport is stimulated by prolactin through alterations in Ctr1 and Atp7A localization

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Milk copper (Cu) concentration declines and directly reflects the stage of lactation. Three Cu-specific transporters (Ctr1, Atp7A, Atp7B) have been identified in the mammary gland; however, the integrated role they play in milk Cu secretion is not understood. Whereas the regulation of milk composition by the lactogenic hormone prolactin (PRL) has been documented, the specific contribution of PRL to this process is largely unknown. Using the lactating rat as a model, we determined that the normal decline in milk Cu concentration parallels declining Cu availability to the mammary gland and is associated with decreased Atp7B protein levels. Mammary gland Cu transport was highest during early lactation and was stimulated by suckling and hyperprolactinemia, which was associated with Ctr1 and Atp7A localization at the plasma membrane.

Using cultured mammary epithelial cells (HC11), we demonstrated that Ctr1 stains in association with intracellular vesicles that partially colocalize with transferrin receptor (recycling endosome marker). Atp7A was primarily colocalized with mannose 6-phosphate receptor (M6PR; late endosome marker), whereas Atp7B was partially colocalized with protein disulfide isomerase (endoplasmic reticulum marker), TGN38 (trans-Golgi network marker) and M6PR. Prolactin stimulated Cu transport as a result of increased Ctr1 and Atp7A abundance at the plasma membrane.

Although the molecular mechanisms responsible for these posttranslational changes are not understood, transient changes in prolactin signaling play a role in the regulation of mammary gland Cu secretion during lactation.

Atp7B; lactation; copper transporters

Copper (Cu) plays an essential role as a cofactor for enzymes that generate cellular energy, cross-link connective tissue and mobilize cellular iron (28). Although fetal Cu stores are high and are mobilized to provide Cu during the early neonatal period (27), adequate dietary Cu intake is essential during the newborn period, as mice suckled from dams with insufficient milk Cu levels die before weaning (35). Normally, milk Cu concentration is high during early lactation but declines as lactation continues (15). This is likely a direct result of the temporal synchronization of three main processes: 1) Cu uptake into the secretory mammary epithelial cell (MEC); followed by 2) Cu secretion from MEC into the alveoli lumen of the mammary gland for sequestration in milk; and then 3) milk release in response to suckling (31). Suckling then stimulates the transient secretion of lactogenic hormones, such as oxytocin and prolactin (30), which initiates milk production for the subsequent feeding. However, the molecular mechanisms that integrate mammary gland Cu uptake and secretion into milk are not well understood.

Mammals have developed specialized transport mechanisms to tightly regulate cellular Cu level, and three Cu-specific transport proteins have thus far been identified in the mammary gland (1, 16, 29). The first step in milk Cu secretion is uptake into the MEC from maternal circulation. Cu transporter-1 (Ctr1), is an essential Cu import protein that imports Cu$^{+2}$ with high affinity (26, 43) and is believed to require multimerization of several Ctr1 proteins (25), possibly forming a membrane channel (21) or a transporter-channel hybrid (3). We have previously determined that mammary gland Ctr1 is localized to both plasma and intracellular membranes (16), which suggests that Ctr1 in the MEC may be cycled within endosomal vesicles and possibly degraded as has been observed in other cell types (21, 34).

Once inside the cell, Cu delivery to secretory pathways is mediated by the metallochaperone Atox1 (11), which interacts directly with both the Menkes (Atp7A) and Wilson (Atp7B) Cu, ATPase export proteins to facilitate Cu exchange. Atp7A belongs to the P-type ATPase family of transmembrane proteins and presumably transports cytoplasmic Cu into the trans-Golgi network or across the plasma membrane (23) and mutations in the Atp7A gene are associated with impaired cellular Cu export and severe Cu deficiency (6). In nonlactating mammary gland, Atp7A is localized to both perinuclear and vesicular compartments (1, 10); however, during lactation, Atp7A is relocalized to the plasma membrane and expression is increased (1). This suggests that mammary gland Atp7A plays an important role in mammary gland Cu secretion during lactation and that it is regulated by as yet unknown transcriptional and posttranslational mechanisms. The Wilson Cu, ATPase (Atp7B) is homologous to Atp7A (29) and transports cytoplasmic Cu into the trans-Golgi network specifically for incorporation into newly synthesized ceruloplasmin (Cp) in the liver (24) and brain (4). Cp is also expressed in the mammary gland (8, 14), and expression increases during pregnancy and markedly increases in conjunction with lactation (5). It is found in the milk of many mammals and milk Cp concentration parallels the decline in milk Cu concentration (5, 20) during lactation. A point mutation in the Atp7B gene that substitutes a valine for a conserved methionine (41) has been identified in the toxic milk mouse (tx), which results in Atp7B mislocalization and reduced Cu-transporting activity. Phenotypically, low milk Cu levels ensue and eventually result in neonatal death from severe Cu deficiency by midlactation (29), suggesting it plays a major role in the transport of Cu into milk, particularly during the early neonatal period.

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In this study, we used the lactating rat and cultured mouse mammary epithelial cells (HC11 cells) to elucidate both longitudinal and hormone-stimulated changes in the localization and expression of mammary gland Ctrl, Atp7A, and Atp7B. Our data suggest that, whereas decreased Atp7B abundance may be responsible for the normal, longitudinal decline in milk Cu concentration, transient elevations in circulating prolactin levels may stimulate Cu secretion into milk by increasing abundance of Ctrl and Atp7A at the plasma membrane in the mammary epithelial cell.

MATERIALS AND METHODS

Experimental animals and tissue collection. This protocol was approved by the Institutional Animal Care and Use Committee at the University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Virgin Sprague-Dawley rats (~250 g) were obtained commercially (Charles River, Wilmington, MA) and fed a control, semipurified experimental diet ad libitum based on AIN-93 recommendations. Rats were maintained in stainless-steel hanging cages on a 12:12-h light-dark cycle. Sprague-Dawley rats (University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Virgin rats were returned to dams to suckle for 30 min. Blood was collected by cardiac puncture into heparinized tubes and separated by centrifugation at 1,500 g for 15 min at 4°C. Rats were killed by asphyxiation with CO₂, and mammary glands were fixed in 4% phosphate-buffered paraformaldehyde or snap frozen in liquid nitrogen and stored at −80°C until analysis of Cu transporter protein levels (by immunoblotting as described below). Serum prolactin was measured using a commercially available kit (Amersham Pharmacia Biotech).

Cu analysis. Plasma, mammary gland, and milk Cu concentration were analyzed by flame atomic absorption spectrophotometry (model Smith-Heifje 4000, Thermo Jarrell Ash, Franklin, MA) as previously described (16).

Quantification of Ctrl, Atp7A, and Atp7B mRNA levels by real-time RT-PCR. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), as previously described (18), and diluted (1 µg/µl) in RNase-free water. RNA integrity was evaluated following electrophoresis through agarose and staining with ethidium bromide (Sigma). cDNA was generated from 1 µg RNA using reverse transcription kit (Perkin Elmer Applied Biosystems, Foster City, CA) following the manufacturer’s instructions, and the reaction was performed at 48°C for 30 min followed by 95°C for 5 min. Gene-specific primers to Ctrl (5’-GGA GAA ATG GCT GGA GCT TT-3’ and 5’-CGG GCT ATG TTG AGT CCT TCA-3’); Atp7A (5’-AGG CCA ACA TAT GAC AAT TAT GAG TGT-3’ and 5’-GTG AAC GAC GAT GTT TGA AGG A-3’); Atp7B (5’-TTG GAA ACC GGG AAT GGA-3’ and 5’-CTG TCA CGT CAG TCA TGT-3’); and GAPDH (5’-TGC CAA GTA TGA TGA CAT CAA GAA G-3’ and 5’-AGC CGA GGA TGC CCT TTA GT-3’). The cDNA was chosen using Primer Express Software (Perkin Elmer Applied Biosystems) and purchased from Qiagen (Valencia, CA). Real-time PCR was performed on 4 µl of the cDNA reaction mixture using the ABI 7900HT real-time thermocycler (Perkin Elmer Applied Biosystems) coupled with SYBR Green technology (Perkin Elmer Applied Biosystems) and the following cycling parameters: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and a final extension and denaturation at 60°C for 15 s and 95°C for 15 s, respectively. The linearity of the dissociation curve was analyzed using the ABI 7900HT software, and the mean cycle time of the linear part of the curve was designated Ct. Each sample was analyzed in duplicate and normalized to GAPDH using the following equation: ΔCtGENE = CtGENE – CtGAPDH. The fold change relative to animals at lactation LD1 was calculated using the following equation: 2ΔΔCtGENE = mean ΔCtGENE at LD1 – ΔCtGENE at LD15. Values represent mean fold change ± SD.

Immunoblotting. Mammary gland (250 mg) was prepared as previously described (18), and total protein concentration was determined by Bradford protein assay (Bio-Rad). Whereas the mammary gland homogenate from stimulated rats consisted primarily of cellular protein, the homogenate from nonstimulated rats was enriched in milk proteins; therefore, Western blot analysis against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) was first performed to normalize samples to cellular protein levels. Equal amounts (100 µg) of cellular protein was resolved by SDS-PAGE (Ctrl, 12%; Atp7A and Atp7B, 7% and Atox1, 16%) and transferred to nitrocellulose at 350 mA for 90 min (Ctrl, Atp7A, Atp7B) or 50 V for 5 h (Atox1). Antibodies to Ctrl, Atp7A, and Atp7B were previously characterized (16). Atox1 antibody was a generous gift from Dr. Jonathan Gitlin, (Washington University School of Medicine). Blots were blocked overnight at 4°C with 5% nonfat milk in PBS/0.1% Tween-20 (PBS-T), washed in PBS-T and incubated with polyclonal antiserum (Ctrl, 1:2,000; Atp7A, 1:5,000; Atp7B, 1:1,500; Atox1 1:1,000) for 45 min. Proteins were detected with donkey, anti-rabbit IgG conjugated to horseshadish...
peroxidase (Amersham Pharmacia), visualized with Super Signal Femto (Pierce), and quantified using the Chemi-doc Gel Quantification System as described previously (18).

**Immunostaining of mammary gland.** Mammary gland was fixed in 4% phosphate-buffered paraformaldehyde for 24 h, washed extensively with PBS and sequentially dehydrated in ethanol. Tissues were embedded in paraffin (Fisher Scientific, Pittsburgh, PA), sectioned (4 μm), and mounted on positively charged microscope slides. Immunostaining was performed, and sections were counterstained with hematoxylin as described previously (18). Non-specific staining was determined following preabsorption of affinity-purified antibody with peptide antigen (1 mg) for 2 h at room temperature before incubation with tissue sections.

**Mammary epithelial cell culture.** Mouse mammary epithelial cells (HC11) were a gift from Dr. Jeffery Rosen (Houston, Texas) and used with permission of Dr. Bernd Gröner (Institute for Biomedical Research, Frankfurt, Germany). HC11 cells were seeded onto glass coverslips (confocal microscopy), polycarbonate dishes (protein isolation), or bicameral cell culture inserts (Cu transport), and grown to confluency in growth medium [RPMI 1640 medium (GIBCO Life Technologies) supplemented with 10% fetal bovine serum, gentamycin, insulin, and EGFr (all from Sigma)] at 37°C with 5% CO₂, as previously described (19). Cells were then cultured in induction medium [growth medium without fetal bovine serum or EGFr containing prolactin (PRL; 1 μg/ml) and dexamethasone (1 μM)] for 48 h to differentiate HC11 cells into a secretary cell type (19). Transepithelial resistance (TEER) was used to monitor tight junction formation across the cell monolayer, and experiments were conducted 4 days post-TEER stabilization.

**Prolactin treatment.** Secreting cells were maintained in induction medium for up to 48 h. To mimic transient PRL stimulation, cells were washed with prewarmed PBS, and then were stimulated with fresh induction medium for up to 1 h at 37°C and 5% CO₂. Following PRL treatment, cells were washed with PBS and sonicated twice for 10 s on ice in lysis buffer [20 mM HEPES, pH 7.4 containing 1 mM EDTA, 250 mM sucrose, and protease inhibitor cocktail (Sigma)]. Samples were clarified by centrifugation at 1,000 g for 5 min, and the protein concentration of the postnuclear supernatant was measured by Bradford assay. Similarly treated cells were used for Cu transport studies.

**Cu transport.** We have previously determined the polarity of HC11 cells (19). To determine effects of PRL stimulation on mammary epithelial cell Cu transport, cells were cultured in bicameral chambers, and Cu transport was assessed following addition of induction medium containing 0.1 μCi ⁶⁷CuCl₂ (8.4 mCi/μg Cu; Oakridge National Laboratory, Oakridge, TN) to the top of the cell culture insert for 1 h at 37°C. Cells were washed extensively with ice-cold PBS and mammary epithelial cell Cu uptake and Cu secretion was determined by quantifying the radioactivity in the cell monolayer and bottom chamber of the bicameral chamber in a gamma scintillation counter.

**Detection of Ctrl, Atp7A, and Atp7B at the plasma membrane by cell surface biotinylation.** To detect the effects of PRL on abundance of Ctrl, Atp7A, and Atp7B at the plasma membrane, confluent HC11 cells were treated with PRL in induction medium for 1 h at 37°C, and then cell surface proteins were biotinylated with Sulfo-NHS biotin (Pierce; 0.5 mg/ml) at 4°C for 1 h. Cells were washed twice with 50 mM glycine, at pH 5 followed by three washes with ice-cold PBS, scraped into cold lysis buffer (50 mM Tris·HCl pH 7.4, 2 mM EDTA, 2 mM EGTA, plus protease inhibitors) and sonicated for 30 s on ice. The crude membrane fraction was pelleted by ultracentrifugation at 150,000 g for 30 min and resuspended in lysis buffer containing NaCl (0.1 M), and membranes were solubilized with SDS (final concentration to 0.2%) at 60°C for 5 min, and then Triton X-100 was added (final to 1%) and briefly sonicated on ice. Insoluble material was pelleted by ultracentrifugation at 100,000 g for 20 min, and supernatant was incubated with 50 μl of a 1:1 slurry of UltraLink-neutravidin beads (Pierce) while rocking at room temperature for 1 h. Beads were pelleted by centrifugation at 2,300 g for 1 min and washed four times with PBS + 1% Triton X-100. Biotinylated proteins were eluted by boiling in SDS-PAGE buffer containing β-mercaptoethanol (5%) and immobiloblotted for Ctrl, Atp7A, or Atp7B as described above. Cells not treated with biotin were used to assess non-specific binding of proteins to Ultralink beads.

**Indirect immunofluorescence and confocal microscopy.** HC11 cells were seeded onto glass coverslips and cultured overnight in growth medium. Cells were treated with PRL (1 μg/ml) in induction medium for 1 h at 37°C. Medium was aspirated, and cells were fixed in 4% paraformaldehyde/PBS, pH 7.4 (methanol for TGN38) for 10 min. Cells were permeabilized with 0.2% Triton X-100/PBS for 10 min. Nonspecific binding was blocked in 5% goat serum/1% BSA in PBS for 20 min followed by incubation with affinity purified antibody (Atp7A, 1 μg/ml; Ctrl1, 5 μg/ml; Atp7B, 5 μg/ml) diluted in blocking solution for 45 min at room temperature. After extensive washing with PBS, primary antibody was detected using Alexa Fluor 488-conjugated goat anti-rabbit IgG diluted in blocking solution (1 μg/ml; Molecular Probes, Eugene, OR) for 30 min at room temperature shielded from light. Cells were extensively washed in PBS and coverslips were drained, mounted in ProLong Gold (Molecular Probes) and sealed with nail polish. Immunofluorescent imaging was performed using a Radiance 2100 Confocal System (Bio-Rad). An Olympus BX50WI microscope with a ×100 UPlanApo oil-immersion lens was used for visualization. Digital images were captured sequentially to eliminate potential interference of fluorochromes, and images were captured using LaserSharp2000, version 4.1 (Bio-Rad) and saved as RGB jpg files to maintain image quality. Colocalization analysis was performed with ImageJ Software (version 1.29X; National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/).

**Subcellular colocalization.** Before fixation, cells were incubated with Alexa Fluor 546-labeled transferrin (recycling endosome marker; Molecular Probes) for 5 min at room temperature, washed twice with PBS, and then fixed with 4% phosphate-buffered paraformaldehyde before immunofluorescent staining of Cu transporters. Colocalization with the endoplasmic reticulum [mouse anti-protein disulfide isomerase (PDI); Affinity Biosystems; 1:100], trans-Golgí network [mouse anti-TGN38, Abcam; 1:200], and late endosomes [mouse anti-mannose 6-phosphate receptor (M6PR) Abcam, 5 μg/ml] were detected with Alexa Fluor 568-conjugated goat, anti-mouse IgG diluted in blocking solution (0.8 μg/ml; Molecular Probes) for 30 min at room temperature.

**Density gradient fractionation.** To assess subcellular localization of Ctrl1, Atp7A, and Atp7B biochemically, cells were cultured in 10-cm² dishes, washed in buffer A (0.25 M sucrose, 10 mM Tris·HCl pH 7.4, 1 mM EDTA and protease inhibitors), carefully scraped into buffer B.
were collected from three independent samples from each experiment. A minimum of three independent times and 10 images per treatment was done in triplicate. All colocalization studies were performed a presented as means \( \pm \) SD relative to LD1. *Significant effect of lactation stage, \( P < 0.05 \).

Fig. 2. Relative protein abundance of Ctrl1, Atp7A, Atp7B, and Atox1 in the mammary gland during early (LD1) and late (LD15) lactation in the rat. Values represent mean protein level \( \pm \) SD relative to LD1. *Significant effect of lactation stage, \( P < 0.05 \).

All statistical comparisons were made using Student’s \( t \)-test, and significance was demonstrated at \( P < 0.05 \).

RESULTS

Plasma, mammary gland, and milk Cu concentrations decrease through lactation. Milk, mammary gland and plasma Cu concentrations were higher during early (E, LD1) compared with late (L, LD15) lactation [milk: 141.5 \( \pm \) 9.0 (E), 26.7 \( \pm \) 3.1 \( \mu \)mol Cu/l (L); mammary gland: 75.4 \( \pm \) 24.6 (E), 24.6 \( \pm \) 9.2 nmol Cu/g wet weight (L); plasma: 41.5 \( \pm \) 6.2 (E), 27.7 \( \pm \) 3.1 \( \mu \)mol Cu/l (L), \( P < 0.05 \)]. These results indicate that the decline in milk Cu concentration could result from mammary gland Cu depletion and/or reduced Cu availability from maternal circulation.

Mammary gland \(^{67}\)Cu transport. To determine longitudinal and suckling-stimulated changes in mammary gland Cu transport, the amount of Cu transported into the mammary gland and milk in suckled and nonsuckled dams during early and late lactation was measured following intravenous administration of \(^{67}\)Cu. Nonsuckled dams transported significantly more Cu into both the mammary gland (30.8 \( \pm \) 7.5 compared with 12.9 \( \pm \) 2.4 nmol Cu, \( P < 0.05 \)) and milk (4.1 \( \pm \) 1.4 compared with 2.0 \( \pm \) 0.2 nmol Cu, \( P < 0.05 \)) during early lactation compared with late lactation, respectively, suggesting that Cu transport from maternal circulation was higher during early lactation and was responsible for the higher mammary gland and milk Cu level. Furthermore, suckling resulted in enhanced Cu transport into both the mammary gland (43.6 \( \pm \) 7.3 and 19.3 \( \pm \) 4.7 nmol Cu, \( P < 0.05 \)) and milk (16.4 \( \pm \) 2.4 and 4.4 \( \pm \) 2.1 nmol Cu, \( P < 0.05 \)) during early and late lactation, respectively. These results suggest that basal mammary gland Cu uptake and secretion was greater during early lactation compared with late lactation and was stimulated \( \sim \)2.5-fold during early lactation by suckling. There was no significant difference in milk volume between early (1.1 \( \pm \) 0.4 ml) and late (1.2 \( \pm \) 0.7 ml) lactation or in inguinal mammary gland weight between early (10.5 \( \pm \) 2.8 g) and late (10.8 \( \pm \) 1.8 g) lactation.

Circulating prolactin concentration. Prolactin concentration was significantly higher during early lactation (170 \( \pm \) 26 ng/ml) compared with late lactation (25 \( \pm \) 2.3 ng/ml), \( P <
0.05, and suckling increased PRL levels (early lactation, 247 ± 21 ng/ml; late lactation, 56 ± 12 ng/ml), \( P < 0.05 \). Rats treated with haloperidol had significantly higher PRL level (650 ± 125 ng/ml) than vehicle-treated rats (156 ± 27 ng/ml), \( P < 0.05 \). These results indicate that suckling stimulated PRL release to a greater extent during early compared with late lactation, which may help to explain enhanced mammary gland Cu transport during this period.

Cu transporter mRNA and protein levels but not localization change during lactation. To determine whether the decline in milk and mammary gland Cu levels was associated with changes in mammary gland Cu transporter expression, we measured relative mRNA (Fig. 1) and protein levels (Fig. 2) of Ctrl1, Atp7A, Atp7B, and Atox1 during early (LD1) and late (LD15) lactation in nonstimulated mammary gland. Results indicate that mammary gland Ctrl1 mRNA and protein levels were not significantly different between early and late lactation, suggesting that higher mammary gland Cu uptake observed during early lactation is not a direct effect of higher Ctrl1 abundance. Although both Atp7A and Atp7B mRNA levels were approximately twofold higher during late compared with early lactation, Atp7A protein paralleled mRNA level while Atp7B protein and mRNA levels were inversely related. The inverse relationship between Atp7B mRNA and protein levels suggests that Atp7B levels may be regulated by reduced translation of Atp7B mRNA or increased degradation of Atp7B protein. Atox1 protein level was significantly higher during late compared with early lactation. This suggests that the decline in milk Cu level may result from reduced Atp7B protein level and is not limited by Cu delivery, as a consequence of low Atox1 abundance, to the secretory pathway. To determine whether the decline in mammary gland and milk Cu levels can be attributed to differences in mammary gland Cu transporter localization, we documented Ctrl1, Atp7A, and Atp7B localization using immunohistochemistry at LD1 and LD15. All three Cu transporters exclusively stained the mammary epithelial cell, as previously described (14), were localized intracellularly, and localization was unaffected by the stage of lactation (Fig. 3). The intracellular localization of Ctrl1 suggests that Ctrl1 may traffic throughout the mammary epithelial cell importing Cu from maternal circulation and/or out of intracellular vesicles into the mammary epithelial cell cytosol. The distribution of Atp7A (throughout the mammary epithelial cell) and Atp7B (restricted to the luminal or apical membrane) indicates that although homologous, Atp7A and Atp7B provide unique, yet possibly complementary functions.

To determine whether suckling-stimulated Cu transport resulted from changes in expression or cellular localization of Ctrl1, Atp7A, and Atp7B, we measured relative Ctrl1, Atp7A, and Atp7B protein levels during early (LD1) and late (LD15) lactation in stimulated mammary gland. We determined that suckling resulted in greater staining of Ctrl1 and Atp7A at the luminal (apical) and serosal (basolateral) plasma membrane domains while Atp7B was unaffected (Fig. 3). Ctrl1, Atp7A, and Atp7B protein levels were all lower in the mammary gland of suckled dams and the electrophoretic mobility of the 30 kDa Ctrl1 protein was decreased, reflecting an increase in estimated molecular mass of ~2 kDa (Fig. 4). To determine whether these suckling-induced changes result from increased PRL levels, we induced hyperprolactinemia in rats using haloperidol. We determined that hyperprolactinemia decreased Ctrl1 and Atp7B protein levels while the Atp7A protein level was
unaffected (Fig. 4). Additionally, both Ctr1 and Atp7A were associated more directly with the plasma membrane in halo-peridol-treated rats (Fig. 3) similar to observations in suckled animals, suggesting that increased mammary gland Cu uptake and secretion, particularly during early lactation, may be a consequence of increased Ctr1 and Atp7A abundance at the plasma membrane, resulting from transient hyperprolactinemia.

Prolactin treatment increases Cu transport and does not affect Ctr1 or Atp7B protein levels but alters Ctr1 and Atp7A localization in HC11 cells. To determine whether PRL treatment directly affects mammary epithelial cell Cu transport, HC11 cells were treated with PRL and Cu transport across a cell monolayer was measured. We determined that PRL significantly stimulated Cu secretion across mammary epithelial cells (2.8 ± 0.1 pmol Cu/cm² compared with 1.9 ± 0.2 pmol Cu/cm² in untreated cells), P < 0.05. To determine whether PRL-stimulated Cu transport in vivo results from increased Cu transporter protein levels or transporter relocalization in mammary epithelia cells, HC11 cells were treated with PRL, and changes in total Cu transporter protein levels and localization were determined by Western blot analysis and immunofluorescent confocal imaging. Although the total abundance of Ctr1 and Atp7A proteins was unaffected, Atp7A protein abundance was slightly lower in PRL-treated cells (Fig. 5), thereby demonstrating that PRL-stimulated Cu transport was not a consequence of increased Cu transporter abundance. To confirm in vivo observations that Ctr1 and Atp7A, but not Atp7B localization, were altered in response to lactogenic stimulation, experiments using confocal microscopy in conjunction with sucrose density fractionation and detection of biotinylated cell surface proteins were conducted. We identified little Ctr1 or Atp7A and no Atp7B at the plasma membrane (Fig. 6) under basal conditions. Similar to observations in the mammary gland, Ctr1 was associated with an intracellular vesicular compartment, a portion of which colocalized with TfR, suggesting endosomal recycling as a potential trafficking mechanism to and from the plasma membrane (Fig. 7). Following lactogenic hormone stimulation, concomitant with increased plasma membrane abundance, the fractions in which Ctr1 was

Fig. 6. Ctr1 and Atp7A protein detected at the plasma membrane and in response to prolactin (1 μg/ml) treatment in HC11 cells. Representative Western blot analysis of total membrane protein extracted from 10-cm² plates (n = 2 plates/treatment), illustrating increased Ctr1 and Atp7A protein abundance at the plasma membrane in response to PRL as detected by cell surface biotinylation. –, Untreated; +, treated.

Fig. 7. Ctr1 is partially colocalized with transferrin receptor in HC11 cells. Intracellular localization of Ctr1 in untreated cells (A) and cells treated with prolactin (B) was detected using affinity purified Ctr1 antibody (6 μg/ml) visualized with Alexa Fluor 488-labeled IgG (Ctr1, green) following incubation with Alexa Fluor 546-labeled transferrin (Transferrin, red) for 5 min at room temperature. Arrows illustrate Ctr1 staining at the cell periphery in PRL-treated cells, which was not detected in untreated cells. Images were collected sequentially at ×100 magnification under oil and merged to illustrate partial colocalization (merge, yellow) of Ctr1 and recycling endosomes; scale bar = 10 μm.

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detected were altered and the primary Ctr1 protein detected by immunoblotting was the largest 70-kDa band that was restricted to two fractions (Figs. 6 and 8). Atp7A was partially co-localized with PDI and M6PR by confocal microscopy (Fig. 9), which was verified by sucrose density fractionation (Fig. 8). Following lactogenic hormone stimulation, the fractions in which Atp7A was detected were dramatically altered and paralleled increased Atp7A abundance at the plasma membrane (Fig. 6). In contrast, Atp7B stained within large punctate vesicles and in partial co-localization with the endoplasmic reticulum (PDI), the secretory compartment (TGN38), and the late endosomal compartment (M6PR) (Figs. 8 and 10). However, lactogenic hormonal stimulation did not alter Atp7B localization or abundance at the plasma membrane (data not shown). These results indicate that suckling-stimulated Cu secretion into milk may result from transient Ctr1 and Atp7A movement primarily within the endosomal pathways in the mammary epithelial cell in direct response to prolactin stimulation.

DISCUSSION

Milk composition changes throughout lactation and for nutrients acquired from maternal circulation, milk concentration is a direct result of the integration of mammary gland uptake and secretion mechanisms, some of which are transiently enhanced in response to suckling (39). The results from this study suggest that the normal decline in milk Cu concentration that occurs during lactation may reflect reduced mammary gland Cu acquisition as a result of declining Cu levels in circulation in combination with decreased Cu secretion into milk from declining mammary gland Atp7B levels. The major findings from this study indicate that, 1) Atp7B may play a constitutive role in milk Cu secretion, whereas 2) Atp7A and Ctr1 facilitate transiently enhanced mammary gland Cu transport in response to lactogenic hormone stimulation (17).

As a secretory cell type, cellular export from the mammary epithelial cell does not necessarily have to occur directly across the plasma membrane, as many milk components, including proteins, lactose, lipids, and some minerals are transported into secretory vesicles that eventually fuse with the luminal membrane for extrusion into milk (39). Thus the punctate staining of Atp7A and Atp7B in the lactating mammary gland indicates that both proteins are associated with intracellular vesicles, suggesting they may traffic through intracellular compartments to facilitate Cu secretion as has been observed in other cell types (7); however, their unique cellular localization and differential responses to suckling and prolactin stimulation suggest they perform distinct roles in mammary epithelial cell Cu secretion. But how does each transporter participate in mammary gland Cu secretion? Our data suggest that mammary gland Cu transport via Atp7B may be a constitutive Cu secretion pathway (4) as Atp7B was always observed proximal to the luminal membrane in lactating mammary gland, similar to reports by Michalczyk et al. (29); mammary gland Atp7B

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**Fig. 8.** Subcellular fractionation of HC11 cells on a sucrose gradient. Fractions (0.5 ml) were collected (1–17) from the top (0.2 M) to the bottom (1.6 M) of a sucrose gradient, and membrane proteins were resolved by electrophoresis and transferred to nitrocellulose. Atp7A, Atp7B, and Ctr1 proteins were detected by Western blot analysis in untreated cells (-PRL) and cells treated with PRL (+PRL). Membranes were stripped and re-probed for PDI (ER marker), trans-Golgi network marker (TGN38), and mannose 6-phosphate receptor (M6PR; late endosome marker) to determine comigration within cytoplasmic compartments. Images illustrate distinct shifts in Atp7A- and Ctr1-associated compartments in response to PRL, whereas there was minimal change observed for Atp7B. Atp7A clearly migrates with PDI and M6PR, whereas Atp7B is associated with fractions containing PDI, TGN38, and M6PR.
abundance parallels milk Cu level; and neither Atp7B localization or expression appears to be distinctly stimulated in vitro or in vivo once a secretory phenotype is established, similar to reports by others (24). The phenotypic observation of significantly reduced milk Cu levels in mice with a mutation in the Atp7B gene further supports this theory. It is important to distinguish the results from this study from observations made by Michalczyk et al. (29) who demonstrated altered Atp7B localization between nonlactating and lactating mammary gland, from the TGN to “a vesicular compartment,” respectively. The focus of our current study was to determine whether Atp7B localization was transiently altered once lactation was established (our data indicate that it is not) and to determine with what “vesicular compartment” Atp7B is associated. Toward this end, in accordance with the observations of Michalczyk et al. (29) and others (37), we detected distinct staining of Atp7B associated with the trans-Golgi network and further documented colocalization within sparse, large vesicles similar to recent observations by Harada et al. (12) in hepatocytes. Our data further identify the late endosomal compartment as the “vesicular compartment” aforementioned. Physiologically, this localization seems reasonable, as the role of Atp7B in the mammary gland may be similar to that in the liver (29, 40), i.e., providing Cu to Cp for export from the cell, as Donley et al. (8) estimated that ~25% of newly secreted Cu in rat milk is Cp associated. We speculate that Cu transported into the late endosomes may be recycled back to the trans-Golgi network by M6PR-containing vesicles, at which point Cu may be incorporated into Cp before secretion into milk through the secretory pathway in mammary epithelial cells.

The lethality of the toxic milk mutation suggests that Cu secreted via Atp7B, perhaps bound to Cp, must be of particular importance during early neonatal life. However, milk Cu concentration is not abolished, and most milk Cu is not Cp associated (7), indicating that non-Atp7B-mediated Cu secretion must play a major role as well. The identification of Atp7A in the mammary gland suggests Atp7A may provide this function, particularly since Atp7A localization is different between lactating and nonlactating mammary gland (1, 2). To further this observation, we have determined that both suckling and hyperprolactinemia was directly associated with increased $^{67}$Cu secretion into milk and the relocalization of Atp7A.
proximal to the plasma membrane during both early and late lactation in vivo. Interestingly, immunohistology suggests that the relocalization of Atp7A in response to suckling may be more distinct during early lactation, and we speculate that this reflects higher circulating PRL levels during this time. Similar to a recent report by Ravia et al. (36), who detected endogenous Atp7A on the apical and basolateral membranes in rat enterocytes, we observed Atp7A staining in association with both the apical (luminal) and basolateral membranes in rat mammary epithelial cells. These results are in contrast to a report by Greenough et al. (9) who used transfected Madin-Darby canine kidney cells and detected Atp7A primarily in association with the basolateral membrane. This suggests some tissue specificity in Atp7A localization or possibly reflects differences in endogenous vs. transfected Atp7A localization, either of which may be the case in our observations of native Atp7A localization in the mammary gland. Nevertheless, these observations are puzzling, as Atp7A has been reported to possess important basolateral membrane dileucine signaling motifs. Removal of the dileucine motif results in apical membrane targeting, indicating that the default trafficking pathway of Atp7A, without basolateral sorting, is to the apical membrane (9). Although purely speculative, perhaps there may be “masking” of the dileucine motif in endogenous Atp7A under certain conditions. Alternatively, other studies have shown that these dileucine motifs actually target proteins to endosomal compartments (38) or to the trans-Golgi network (32), thus they may facilitate the localization of Atp7A within intracellular vesicles to transport Cu into endosomes or the secretory compartment (1, 2). Evidence from this current study now confirms Atp7A colocalization within both the endoplasmic reticulum and late endosomal compartments in cultured mammary epithelial cells. As we identified colocalization of mammary epithelial cell Atp7A with both these compartments, what we may have detected in lactating rat mammary gland was enhanced abundance of Atp7A-containing vesicles at the apical membrane domain. Our data from cultured mammary epithelial cells indicate that prolactin treatment also resulted in increased Cu secretion that was associated with a quantifiable increase in Atp7A abundance at the plasma membrane, likely

Fig. 10. Atp7B is partially colocalized with the trans-Golgi network, late endosomes and the endoplasmic reticulum in secreting HC11 cells. Intracellular localization of Atp7B was detected using affinity purified Atp7B antibody (5 μg/ml) and visualized with Alexa Fluor 488-labeled IgG (Atp7B, green). Colocalization with the trans-Golgi network (TGN38, red), endoplasmic reticulum (PDI, red), and late endosomes (M6PR, red) was detected in permeabilized HC11 cells. Images were collected sequentially and merged to illustrate colocalization (merge, yellow). Images were collected at ×100 magnification under oil; scale bar = 10 μm.
reflecting the dramatic change in intracellular Atp7A localization from its tight perinuclear compartment to diffuse intracellular vesicles. Thus the relocalization of mammary gland Atp7A to the plasma membrane in vivo suggests that Atp7A may be responding directly to prolactin signaling mechanisms or may alternatively reflect alterations in cellular Cu pools (33).

Although intracellular concentration of free Cu is very low and thus there is little need to invoke active transport from maternal circulation into the mammary gland, as a positively charged ion, Cu transport across the plasma membrane must somehow be facilitated. Although it has been suggested that circulating Cp performs this function (13), the possibility that Cp facilitates mammary gland Cu uptake is unlikely. For example, during early lactation, plasma Cu level is high (42), and Cu is primarily bound loosely to albumin and amino acids and has been shown to be directly taken up by the mammary gland (8). In contrast, during late lactation, not only is plasma Cu concentration low but the distribution of Cu in plasma changes to a more Cp-associated pool (5), suggesting that milk Cu levels may actually reflect the availability of “loosely bound Cu” for uptake into the mammary gland. As a result of our previous studies (16) and a recent report by Kuo et al. (22), we propose that mammary gland Cu uptake is mediated by the copper uptake protein Ctrl. Although mammary gland Cu uptake was highest during early lactation and increased in response to suckling, elevated Ctrl abundance does not appear to explain this observation. In fact, Ctrl abundance actually decreased in response to suckling and chemical-induced hyperprolactinemia, potentially as a consequence of negative feedback of elevated prolactin levels on prolactin receptor expression from its tight perinuclear compartment to diffuse intracellular vesicles. Thus the relocalization of mammary gland Atp7A to the plasma membrane in vivo suggests that Atp7A may be responding directly to prolactin signaling.

In summary, the evidence presented here indicates that the mammary gland copper transport system is regulated by periparturient changes to a more Cp-associated pool (5), suggesting that milk Cu levels may actually reflect the availability of “loosely bound Cu” for uptake into the mammary gland. As a result of our previous studies (16) and a recent report by Kuo et al. (22), we propose that mammary gland Cu uptake is mediated by the copper uptake protein Ctrl. Although mammary gland Cu uptake was highest during early lactation and increased in response to suckling, elevated Ctrl abundance does not appear to explain this observation. In fact, Ctrl abundance actually decreased in response to suckling and chemical-induced hyperprolactinemia, potentially as a consequence of negative feedback of elevated prolactin levels on prolactin receptor expression.

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