Riboflavin uptake transporter Slc52a2 (RFVT2) is upregulated in the mouse mammary gland during lactation

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1Physiology and Experimental Medicine Program, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada; and 2Department of Pharmacology and Toxicology, University of Toronto, Toronto, Ontario, Canada; and 3Department of Paediatrics, University of Toronto, Toronto, Ontario, Canada

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Riboflavin uptake transporter Slc52a2 (RFVT2) is upregulated in the mouse mammary gland during lactation. Am J Physiol Regul Integr Comp Physiol 310: R578–R585, 2016. First published January 20, 2016; doi:10.1152/ajpregu.00507.2015.—While it is well recognized that riboflavin accumulates in breast milk as an essential vitamin for neonates, transport mechanisms for its milk excretion are not well characterized. The multidrug efflux transporter Abcg2 in the apical membrane of milk-producing mammary epithelial cells (MECs) is involved with riboflavin excretion. However, it is not clear whether MECs possess other riboflavin transport systems, which may facilitate its basolateral uptake into MECs. We report here that transcripts encoding the second (Slc52a2) and third (Slc52a3) member of the recently discovered family of Slc52a riboflavin uptake transporters are expressed in milk fat globules from human breast milk. Furthermore, Slc52a2 and Slc52a3 mRNA are upregulated in the mouse mammary gland during lactation. Importantly, the induction of Slc52a2, which was the major Slc52a riboflavin transporter in the lactating mammary gland, was also observed at the protein level. Subcellular localization studies showed that green fluorescent protein-tagged mouse Slc52a2 localized mainly to the cell membrane, with no preferential distribution to the apical or basolateral membrane in polarized kidney MDCK cells. These results strongly implicate a potential role for Slc52a2 in riboflavin uptake by milk-producing MECs, a critical step in the transfer of riboflavin into breast milk.

EXCLUSIVE BREASTFEEDING for the first 6 mo is associated with significant health benefits to the nursing infant, including better neurodevelopmental outcomes and reduced risk of infection, allergies, metabolic disorders, and gastrointestinal morbidities (23). Breast milk is a complex mixture of macronutrients (e.g., proteins, carbohydrates, and lipids)—some of which function as bioactive factors—and micronutrients such as vitamins (1). Despite our extensive knowledge of the nutritional composition of breast milk, we still have a very poor understanding of transport mechanisms responsible for transferring many of these nutrients into breast milk and reasons for individual variations (17).

One important example is the water-soluble vitamin, riboflavin (i.e., vitamin B2). Riboflavin and its bioactive coenzyme derivatives flavin adenine dinucleotide and flavin mononucleotide are important for redox reactions in energy metabolism

(20). We have known for decades that riboflavin is highly concentrated in breast milk (see Ref. 19 for a historical perspective) and that maternal riboflavin status can impact riboflavin levels in breast milk (2, 18, 21). Despite this, it was not until more recently that mammary transport mechanisms for riboflavin were discovered.

The multidrug efflux transporter breast cancer resistance protein, encoded by Abcg2, is the principal mechanism for transferring riboflavin and its substrate xenobiotics into breast milk. This is based on the observation that Abcg2 knockout mice lack the ability to actively secrete riboflavin into breast milk and, as such, have ~60-fold lower levels of riboflavin in breast milk (8). Given that Abcg2 is expressed in the apical/luminal membrane of mammary epithelial cells (MECs) (11, 27), basolateral uptake mechanisms must exist to efficiently deliver riboflavin into milk-producing MECs. However, these basolateral uptake mechanisms have not yet been characterized.

Recently, three riboflavin uptake transporters have been discovered (28, 29, 31). These transporters, known as RFVT1 (RFT1), RFVT2 (RFT3), and RFVT3 (RFT2), are encoded by Slc52a1, Slc52a2, and Slc52a3, respectively (see Table 1 and Ref. 30). Slc52a1 shows both a low and limited expression profile in human tissues, whereas in most tissues, the expression of Slc52a3 predominates (29). Slc52a3 is highly expressed in the human small intestine, and cellular localization studies showed that green fluorescent protein (GFP)-tagged human Slc52a3 is mainly localized to the apical membrane in polarized kidney MDCK cells, suggesting a role in intestinal riboflavin absorption (25, 26, 29). In contrast, GFP-tagged human Slc52a1 and Slc52a2 are mainly localized to the basolateral membrane. Genetic mutations that alter the transport activity or gene expression of Slc52a2 (4, 7, 9) and Slc52a3 (5, 6, 10) are associated with Brown-Vialetto-Van Laere syndrome, a very rare neurodegenerative disorder characterized by pontobulbar palsy and sensorineural deafness (22).

In the present study, we sought to determine whether these newly discovered riboflavin uptake transporters are expressed in the mammary gland and whether their level of expression changes at different stages of mammary gland development.

MATERIALS AND METHODS

Cell lines and reagents. Human embryonic kidney (HEK 293) cells were obtained from the American Type Culture Collection and grown in DMEM supplemented with 10% FBS. HC11 mouse MECs were a gift from Dr. Jason Matthews (Department of Pharmacology and Toxicology, University of Toronto) and were grown as described
SLC52A2 expression in mouse mammary gland

Previously (27), Madin-Darby canine kidney (MDCK) cells were a gift from Dr. Clifford Lingwood (Molecular Structure & Function Program, Sickkids Research Institute) and were grown in DMEM supplemented with 10% FBS (14). Cells were maintained in a 5% CO2 humidified incubator at 37°C. Tissue culture reagents were from Wisent (Montreal, QC, Canada). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Unless specified, all other reagents were purchased from Life Technologies (Burlington, ON, Canada).

Antibody against mouse SLC52A2. An antibody against mouse SLC52A2 was generated through the Thermo Scientific Pierce Custom Antibody Services. A unique peptide sequence (amino acid 433-565) in the COOH terminus of mouse SLC52A2 was conjugated to keyhole limpet hemocyanin and injected into a rabbit. Booster shots were given on days 14, 42, and 56. Affinity-purified antibodies from serum samples collected on day 7 were aliquoted and stored as a working stock at −20°C or at −80°C for long-term storage. This polyclonal antibody preparation denoted herein as anti-mSlc52a2.d72 was used for all Western blot experiments.

Animals. Female FVB and C57BL/6 mice, originally from Jackson Laboratory (Bar Harbor, ME) and Charles River Laboratories (Wilmington, MA), respectively, were bred in-house at the Toronto Centre for Phenogenomics (TCP). Animals were given standard chow containing 15 mg/kg riboflavin (Teklad 2018, Harlan Laboratories) and water ad libitum, and they were housed with alternating 12:12-h light-dark cycles. Virgin mice (9–10 wk old) were mated to obtain early pregnant (15.5 day gestation) and lactating (1–2 wk) mice. Mice at various stages of mammary gland development were not age-matched, but all mice used were between 9 and 14 wk old. All procedures were approved by the TCP Animal Care Committee and are in accordance with Canadian Council on Animal Care guidelines.

Human breast milk. Otherwise discarded breast milk samples were obtained under a waiver from the Research Ethics Board at the Hospital for Sick Children. Deidentified breast milk (~15 ml) donated by three healthy mothers (1-2 mo postpartum), who gave birth to full-term newborns, was collected using a standard breast pump. Milk was immediately processed or stored at 4°C and processed within 6 h. Approximately 15 ml of milk was centrifuged at 3,000 rpm for 10 min at 4°C, and the upper fat layer (milk fat globule, MFG) was transferred into a new tube. Milk fats were treated with 1 ml of TRizol and then stored at −80°C until processed for RNA isolation. Milk samples were processed as individuals and were not pooled for analysis.

Gene expression analysis. RNA was isolated from cells, tissues, or milk fat, using TRIzol (Life Technologies) or QIAzol (Qiagen, Valencia, CA), as per the manufacturer’s instructions. RNA pellets were dissolved in DEPC-treated H2O, quantitated using NanoDrop 2000 spectrophotometer, and stored at −80°C. Total RNA (2 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies) and oligo(dT)16. Real-time RT-PCR was used to quantify gene expression by amplifying 100 ng cDNA from a 20-μl reaction with TaqMan Universal PCR Master Mix, No AmpErase UNG and inventoried TaqMan primer/probe sets (Applied Biosystems) on an ABI 7500 system. TaqMan primer/probe sets used were as follows: SLC52A2 (Hs00363824_g1), SLC52A3 (Hs00364295_m1), ACTB (Hs99999903_m1), Slc52a2 (Mm01205717_g1; Mm00518631_g1), Slc52a3 (Mm00510191_g1), Abcg2 (Mm00496364_m1), Krt18 (Mm01601704_g1), and U6 (Mm00449156_m1). Relative mRNA expression was calculated using the 2−ΔΔCt method, normalizing to the expression of ACTB/Actb, Krt18, or U6, where indicated. For absolute quantitation, transcript abundance was determined using

Table 1. Gene nomenclature and common alternative gene names of riboflavin uptake transporters

<table>
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<tr>
<th>Gene</th>
<th>Human</th>
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<td>SLC52A1</td>
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<td>SLC52A2</td>
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<td>SLC52A3</td>
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Fig. 1. Riboflavin transporters are upregulated in the lactating mouse mammary gland. Relative mRNA expression of riboflavin transporters Slc52a2, Slc52a3, and Abcg2 in FVB (A) and C57BL/6 (B) mice. Gene expression was normalized to Krt18 mRNA and then presented as fold change over virgin animals, means ± SD. A: ***P < 0.001, one-way ANOVA, Dunnett’s post hoc pairwise comparison to virgin (n = 5-9 per group). B: ***P < 0.001, Student’s t-test (n = 4 virgin, 7 lactation). C: absolute quantitation of Slc52a2 and Slc52a3 mRNA in lactating mouse mammary glands. Results shown are copies per microgram CDNA, means ± SD; n = 5 per group. **P < 0.01, ***P < 0.001, paired t-test.
293 cells were seeded at 3 × 10⁴ for MDCK cells. To test the specificity of anti-mSlc52a2.d72, HEK 3:1 Fugene HD (Roche) to DNA ratio, whereas a 5:1 ratio was used (Qiagen) ON, Canada) and prepared for transfection using HiSpeed Maxi-Prep Kit (Thermo Scientific, Waltham, MA), respectively, and transfected with 250 ng of plasmid per chamber. For riboflavin uptake experiments, HEK 293 cells were seeded at 3 × 10⁵ cells per well on 12-well poly-d-lysine-coated plates (Corning, Corning, NY) and transfected with 1 μg of plasmid per well.

**Preparation of cell and tissue lysate.** The QProteome cell compartment kit (Qiagen) was used to isolate subcellular fractions from transfected HEK 293 cells. Whole cell and tissue lysates were prepared as previously described (27). Briefly, whole cell lysates were prepared from transfected cells using RIPA buffer. Tissue lysates were prepared by homogenizing 20 mg of snap-frozen tissue in 200 μl RIPA buffer using a Polytron 2100 set at 15,000 rpm. Protein concentration was determined using the Bradford Assay.

**Western blot analysis.** Whole cell lysates (20 μg), subcellular fractions (2 μg or 20 μg), and tissue lysates (40 μg) were resolved in 4–12% Bis-Tris gels and transferred to Hybond-C nitrocellulose membranes (GE Healthcare) using the NuPage Novex System (Life Technologies, Carlsbad, CA). Whole cell lysates and tissue lysates were prepared as previously described (27). Briefly, whole cell lysates were prepared from transfected cells using RIPA buffer. Tissue lysates were prepared by homogenizing 20 mg of snap-frozen tissue in 200 μl RIPA buffer using a Polytron 2100 set at 15,000 rpm. Protein concentration was determined using the Bradford Assay.

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SLC52A2 expression in mammalian tissues

**RESULTS**

**SLC52A riboflavin transporter transcripts are present in the human milk fat globule.** To determine whether riboflavin transporters are expressed in mammary glands during lactation, we first examined the mRNA expression of SLC52A1, SLC52A2, and SLC52A3 in MFGs isolated from breast milk samples of three women. The MFG in breast milk represents an accessible source of RNA that reflects the gene expression profile in mammary gland tissues and epithelial cells (3). Despite high interindividual variations, SLC52A2 and SLC52A3 transcripts were detected in the MFG of all three subjects (2.75 ± 4.35% and 0.04 ± 0.04% relative to β-actin, respectively), whereas SLC52A1 was undetectable.

**Riboflavin uptake assay.** Forty-eight hours after transfection, cells were fixed with 10% neutral buffered formalin and then washed with PBS supplemented with 100 mM glycine. Cells were permeabilized with 0.3% vol/vol Triton X-100, 0.05% vol/vol Tween-20, and then incubated with rat anti-β-actin (1:100; cat. MAB1378; EMD Millipore) overnight at 4°C. After a brief wash with PBS, cells were incubated with goat anti-rat Cy3 (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Subsequently, cells were washed with PBS, incubated with 5 μM Draq5 (Cell Signaling Technology, Danvers, MA) for 2 h at room temperature and then mounted with ibidi mounting medium. Spinning disk confocal images were acquired using an Olympus IX81 microscope with ×60 objective and processed using Velocity 6.3 (PerkinElmer, Waltham, MA).

**Immunocytochemistry and confocal microscopy.** Forty-eight hours after transfection, HEK 293 cells were preincubated with N1 buffer (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM K₂HPO₄, 5.6 mM D-glucose, pH 7.5) for 1 h at 37°C, and then incubated with N1 buffer containing 10 nM or 100 nM [3H]-riboflavin for 10 min at 37°C. The uptake process was terminated by placing the plates on ice and washing the cells twice with ice-cold PBS. Cells were lysed with 1 ml 0.01 N NaOH for 1 h at 37°C. The radioactivity present in 600 μl of cell lysate was determined using a liquid scintillation counter (Beckman Coulter LS6500). Protein content in the cell lysate was measured using the bicinchoninic acid assay (Thermo Scientific).

**Statistical analysis.** All experiments were performed at least three times and expressed as means ± SD, unless stated otherwise. Results were analyzed using GraphPad Prism 6 (La Jolla, CA), with a P < 0.05 considered statistically significant.

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**Statistical analysis.** All experiments were performed at least three times and expressed as means ± SD, unless stated otherwise. Results were analyzed using GraphPad Prism 6 (La Jolla, CA), with a P < 0.05 considered statistically significant.
Protein expression of SLC52A2 is induced in the lactating mammary gland. We generated an antibody (anti-mSlc52a2.d72; see MATERIALS AND METHODS) against a unique peptide sequence in the COOH terminus of mouse SLC52A2, the predominant riboflavin uptake transporter expressed in lactating mammary glands. Western blot analysis showed that anti-mSlc52a2.d72 detected two bands at the predicted molecular weight of mouse SLC52A2 (~47 kDa) in whole cell lysates of HEK 293 cells transfected with pCMV6-mSlc52a2 encoding mouse SLC52A2 (Fig. 3A). Both bands were absent in cells transfected with empty plasmid, demonstrating that this antibody recognized mouse SLC52A2. We suspect that the heavier band may be glycosylated SLC52A2, since a predicted N-glycosylation site is present at amino acid position 178 (www.uniprot.org, UniProtKB/Swiss-Prot database, accession no. Q9D8F3). We further tested the specificity of our antibody in tissue lysates isolated from the mammary glands of lactating mice, which express Slc52a2 transcripts. Indeed, anti-mSlc52a2.d72 detected a strong signal at the expected molecular...
weight of mouse SLC52A2, which was competed out by preincubating anti-mSlc52a2.d72 with free synthetic peptide (Fig. 3B). It is noteworthy that anti-mSlc52a2.d72 also detected a lower molecular weight band (~25 kDa) that was also competed out with free peptide. On the basis of molecular weight alone, we assume that it is not mouse SLC52A2, although its identity is unknown.

Using our custom antibody, we next examined the protein expression of SLC52A2 in other mouse tissues (Fig. 4A). An immunoreactive band corresponding to SLC52A2 was observed in the kidney, heart, brain, colon, small intestine, and liver. Interestingly, SLC52A2 showed decreasing expression from the proximal to distal small intestine. Among all tissues examined, the lactating mammary gland expressed the highest levels of SLC52A2. Importantly, this level of expression was higher than that observed in virgin mammary glands (Fig. 4B), consistent with the lactation-associated induction of Slc52a2 mRNA. In contrast, there were no obvious changes to SLC52A2 protein expression in the small intestine and in the liver between virgin and lactation (Fig. 4C), which is also consistent with the mRNA results.

Mouse SLC52A2 is localized to both the apical and basolateral membranes. Partly because of the presence of a strong unidentifiable band that could be competed out with free SLC52A2 peptide, immunohistochemistry with anti-mSlc52a2.d72 was inconclusive to determine the subcellular localization of mouse SLC52A2 protein (data not shown). Therefore, we generated an expression plasmid (pAcGFP-mSlc52a2) with GFP tagged to the NH2 terminus of mouse SLC52A2. In transfected HEK 293 cells, unlike GFP, which was expressed only in the cytoplasm, GFP-tagged SLC52A2 showed clear predominant localization to the cell membrane, as demonstrated by the colocalization of the GFP signal with the transmembrane protein α-6 integrin (Fig. 5A). Similar results were observed by Western blot analysis of isolated cytoplasmic and crude membrane fractions (Fig. 5B). Importantly, the untagged form of mouse SLC52A2 remained predominantly membrane localized when overexpressed in HEK 293 cells. This suggests that membrane localization of GFP-tagged SLC52A2 was not an artefact of the GFP-tag (Fig. 5C). Furthermore, overexpression of either untagged (Fig. 5D) or GFP-tagged (Fig. 5E) mouse SLC52A2 increased riboflavin uptake in HEK 293 cells, indicating that GFP-tagged SLC52A2 is functional. Consistent with the HEK 293 results, GFP-tagged SLC52A2 also mainly localized to the plasma membrane of HC11 mouse mammary epithelial cells (Fig. 5F).

We further investigated whether mouse SLC52A2 preferentially localized to a certain membrane compartment in polarized canine kidney MDCK cells (Fig. 5G). Z-stacked confocal images revealed that GFP-tagged mouse SLC52A2 not only colocalized with the basolateral membrane marker α-6 integrin, but it was also expressed at the apical membrane.

DISCUSSION

While it is well documented that milk is a rich source of riboflavin, it is not entirely clear how riboflavin enters and accumulates in breast milk. Recently, others have found that apically localized ABCG2 play an important role in the excretion of riboflavin into breast milk (8). However, specific basolateral uptake mechanisms have yet to be characterized.

We report here for the first time that riboflavin uptake transporters are upregulated in the mouse mammary gland during lactation (Figs. 1 and 4B). Although both Slc52a2a and Slc52a3 are upregulated during lactation, Slc52a2 is the predominant Slc52a riboflavin transporter expressed in lactating mouse mammary glands (Fig. 1C). This is in agreement with published RNA-sequencing data of the human milk fat globule transcriptome, which showed that SLC52A2 was at least 100-fold more abundant than SLC52A1 or SLC52A3 (15), suggesting that this may be a conserved expression profile across species.

We further show that SLC52A2 protein is expressed rather ubiquitously in various normal mouse tissues (Fig. 4A), with a particularly high expression in the mammary glands during lactation. Interestingly, there were segmental differences in SLC52A2 expression in the small intestine, with the level of expression decreasing toward the distal small intestine. This trend contrasts that of Slc52a3, which is expressed highest in the ileum (i.e., distal part of the small intestine) (32).

In the multiple cell lines examined, although GFP-tagged mouse SLC52A2 clearly showed predominant localization to the cell membrane, some GFP signal was also observed within the cytoplasm. This is consistent with previous reports for human SLC52A2 that expression was often observed within intracellular vesicles (24, 26). The biological role of these vesicular localized SLC52A2 remains to be examined but may
simply represent intermediaries in the trafficking of SLC52A2 between subcellular compartments.

In the present study, we showed that mouse SLC52A2 was localized to both apical and basolateral membranes in polarized MDCK cells, a canine renal tubular epithelial cell line (Fig. 5G). Previously, others have demonstrated that human SLC52A2 is primarily localized to the basolateral membrane in Caco-2 and MDCK cells (26). Un fortunately, mammalian epithelial cell lines in various culture conditions do not represent fully polarized lactating mammary gland, and as such, we were not able to investigate subcellular localization of SLC52A2 in a more relevant system. It will be very important in future studies to confirm whether SLC52A2 is localized to both apical and basal membranes of the lactating mammary gland epithelia.

Perspectives and Significance

Taken together, the emerging picture is that SLC52A2, which is expressed in the lactating mammary glands of humans and mice, facilitates delivery of riboflavin from the maternal circulation into milk-producing MECs (Fig. 6). Once inside MECs, riboflavin is efficiently pumped into the milk by ABCG2, where some of the riboflavin may become bound to milk proteins (12, 13, 16) and, therefore, in spite of apical uptake transporters, accumulates in breast milk. Our findings suggest that it would be important to determine whether polymorphisms that reduce the function of these riboflavin uptake transporters (4–7, 9, 10) will also reduce riboflavin concentration in breast milk since inadequate levels of riboflavin in breast milk proteins (12, 13, 16) and, therefore, in spite of apical uptake transporters, accumulates in breast milk. Our findings suggest that it would be important to determine whether polymorphisms that reduce the function of these riboflavin uptake transporters (4–7, 9, 10) will also reduce riboflavin concentration in breast milk since inadequate levels of riboflavin in breast milk could result in riboflavin deficiency in the nursing infant.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


