BioMol Concepts, Vol. 3 (2012), pp. 581–591 • Copyright © by Walter de Gruyter • Berlin • Boston. DOI 10.1515/bmc-2012-0025

Review

Milk lipid secretion: recent biomolecular aspects

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Abstract

Neonates of most species depend on milk lipids for calories, fat-soluble vitamins, and bioactive lipid components for growth and development during the postnatal period. To meet neonatal nutrition and development needs, the mammary gland has evolved efficient mechanisms for synthesizing and secreting large quantities of lipid during lactation. Although the biochemical steps involved in milk lipid synthesis are understood, the identities of the genes mediating these steps and the molecular physiology of milk lipid production and secretion have only recently begun to be understood in detail through advances in mouse genetics, gene expression analysis, protein structural properties, and the cell biology of lipid metabolism. This review discusses emerging data about the molecular, cellular, and structural determinants of milk lipid synthesis and secretion within the context of physiological functions.

Keywords: cellular mechanisms; endoplasmic reticulum; milk lipid; molecular model; secretion.

Introduction

Milk lipids are a major source of the calories needed for neonatal growth in most species (1), and provide a mechanism for delivery of fat-soluble vitamins and essential fatty acids to developing infants. Observations that the lipid content of milk can vary from around 50% (w/v) in fur seals and other aquatic mammals to slightly more than 1% in donkeys (www.havemilk.com) suggest that the processes regulating milk lipid production have been conditioned by evolution to meet species-specific nutritional needs of offspring. In species where it has been studied, it is estimated that milk lipid production makes significant demands on maternal metabolism, requiring adaptations in nutrient intake, trafficking, and utilization (2). In humans and rodents, it is thought that during lactation the mammary gland is one of the most active lipid-synthesizing and lipid-secreting organs in the body (2–4). To meet milk lipid production demands, the mammary gland must be able to efficiently synthesize, transport, and secrete triglycerides (TAG). Because these processes are induced during pregnancy as the mammary gland develops into a secretory organ (5), and they represent robust functions of the mammary gland during lactation, the mammary gland has long represented an important model for investigating the biochemistry, cell biology, and physiology of lipid metabolism. Although much of the general framework of milk lipid synthesis and secretion has been known for years (6), the cellular and molecular mechanisms mediating these functions have only recently begun to be uncovered. This review focuses on advances in understanding the molecular and cellular processes governing synthesis and secretion of milk lipids, and conceptual insights gained from these studies.

Mechanisms of milk lipid production

Milk lipids are derived by secretion of cytoplasmic lipid droplets (CLD). These organelle-like structures, which are found in most cell types, function in the regulation of neutral lipid storage, hydrolysis, and trafficking, and there is increasing evidence that they play important roles in controlling cellular lipid homeostasis (7). Many of the basic structural features of CLD are similar to those of serum lipoproteins. They are composed of a neutral lipid core, which in mammary epithelial cells is >95% triacylglycerol. Like lipoprotein particles, their neutral lipid core is surrounded by a monolayer of phospholipids, and coated by specific classes of proteins that serve structural and functional roles (8).

Extensive evidence from the study of the mammary gland and other tissues indicate that CLD originate from the endoplasmic reticulum (ER), by processes that as yet are not fully understood (6, 9–12). The prevailing view is that CLD originate by budding of neutral lipids that accumulate as lens-type structures between ER membrane leaflets (10). Although this mechanism appears to account for the known structural organization of CLD, neither lens-like structures nor budding CLD have been unequivocally observed (11), even in highly lipogenic organs, such as the mammary gland (unpublished observations). An alternative view is that neutral lipids are synthesized in specialized ER-cytoplasmic domains that form ‘egg cup-like’ structures (11). Three-dimensional freeze-fracture studies documenting the presence of lipid droplets partially enveloped by ER membranes (11) support this model; however, the model does not entirely account for the known structural features of CLD. A variation of the ‘egg cup’

model suggests that CLD originate from interactions of ER loops containing neutral lipid synthesis domains (12). Like the 'egg cup' model, this model can account for some but not all of the structural features of CLD.

Despite these limitations, neutral lipids are known to be synthesized by ER enzymes (Figure 1) (13–15), and ultrastructural and proteomic studies have demonstrated that CLD directly contact ER membranes (16, 17). Moreover, newly synthesized TAG have been shown to be rapidly incorporated into ER-associated CLD in lactating mammary tissue (9) and *de novo* generation of CLD has been demonstrated in ER-microsome fractions isolated from lactating mammary glands (18).

Significantly, there is marked ER expansion during mammary gland differentiation (19–21), and in species in which it has been studied, the ER accounts for approximately 25% of the total volume of mammary epithelial cells in lactating animals (21). Thus, CLD production by ER processes may represent a regulatory step in the overall rate by which lipids are transferred into milk. Loss- and gain-of-function studies have identified TAG synthesis and stabilization, as well as *de novo* fatty acid synthesis, as critical physiological regulators of CLD and milk lipid production.

TAG synthesis

The TAG core of CLD is synthesized by sequential fatty acid esterification of glycerol-3-phosphate (22) or sn-2-monoacylglycerol (23), by enzymes located in the ER (Figure 1). The final step, esterification of diacylglycerol (DAG) to form TAG, is catalyzed by diacylglycerol *O*-acyltransferase (DGAT). Two genetically different DGAT isoforms (DGAT1, DGAT2), with different physiological functions, are present in mammalian tissues (24, 25). DGAT1 appears to be specifically responsible for TAG synthesis by mammary epithelial cells (Figure 1). DGAT1-null mice are viable and possess significant amounts of white adipose tissue; however, they have altered metabolic properties, defects in hair growth, and fail to lactate (26). Histological and electron microscopic analyses showed that the development of their mammary glands was impaired, and that CLD were absent from their mammary epithelial cells (26, 27). Through transplantation experiments, it was shown that loss of DGAT1 in the stromal compartment of the mammary gland was responsible for glandular development defects. DGAT1 loss in mammary epithelial cells, however, did not appear to affect or impair mammary gland development but did result in failure to form CLD in mammary epithelial cells and undergo functional differentiation (27). Evidence in cattle showing that catalytic activity enhancing mutations in DGAT1 are associated with increased milk lipid content further implicates TAG synthesis as a rate-limiting step in CLD and milk lipid production (28).

Synthesis of DAG in mammary epithelial cells occurs by the glycerol-3-phosphate pathway. Members of the glycerol-3-phosphate acyltransferase (GPAT) family catalyze the first acylation step in this process, the formation

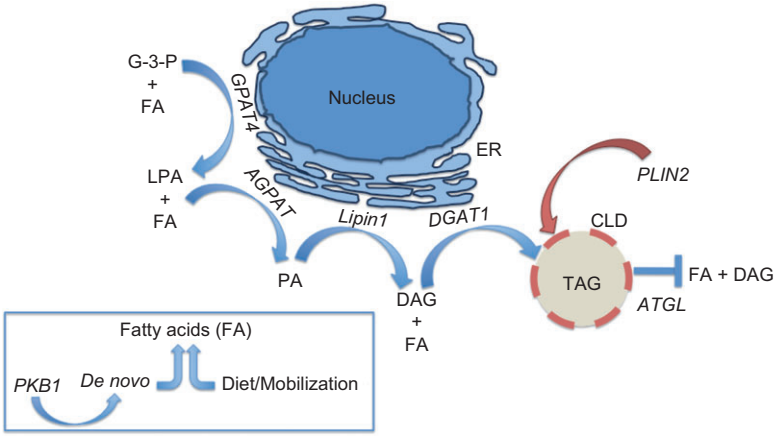

Figure 1 Molecular determinants of milk lipid formation.
The immediate precursors of milk lipids are CLD. These organelle-like structures, which originate from specific ER domains, are composed of a TAG core that is surrounded by a phospholipid monolayer and coated with functionally specialized proteins. Perilipin 2 (PLIN2, indicated by the red dashed line surrounding the TAG core) is the most abundant CLD-associated protein in milk-secreting cells. By inhibiting adipose triglyceride lipase (ATGL)-mediated lipolysis of TAG to fatty acids (FA) and diacylglycerol (DAG), PLIN2 plays an essential role in stabilizing CLD. The TAG core is synthesized by sequential FA acylation of glycerol by four ER membrane-associated enzymes. The first step in TAG synthesis, the addition of FA to glycerol-3-phosphate (G-3-P) to form LPA, is specifically catalyzed by glycerol-3-phosphate-*O*-acyltransferase-4 (GPAT4). Loss of GPAT4 prevents milk lipid formation and lactation. The addition of FA to LPA to form PA is catalyzed by, as yet undefined, members of the 1-acylglycerol-3-phosphate-*O*-acyltransferase (AGPAT) family. Lipin1 catalyzes the removal of the phosphate group from PA to form DAG. The final step in TAG synthesis, the addition of FA to DAG, is specifically catalyzed by diacylglcyerol-*O*-acyltransferase-1 (DGAT1). Loss of DGAT1 prevents milk lipid formation and lactation. Inset: The FA substrates for synthesis of milk lipids are derived from the diet, by mobilization from adipose stores or by *de novo* synthesis from glucose. Protein kinase-B1 (AKT1) regulates the *de novo* synthesis pathway, and disruption of its functions is associated with abnormalities in milk lipid production.

of lysophosphatidic acid (LPA) from glycerol-3-phosphate (Figure 1) (29). Mammals express four GPAT isoforms (29). However, only GPAT4 appears to play a physiologically significant role in catalyzing glycerol-3-phosphate acylation to form milk lipids. Transcript levels of this isoform increase in mammary glands of mice and cattle in response to lactation (30, 31); in mice, loss of GPAT4 (which was initially identified as AGPAT6) is associated with the nearly complete absence of lipids in milk and the absence of CLD in mammary epithelial cells (30, 32), with apparently negligible effects on adipose tissue lipids (30).

Less is known about the identities of the enzymes catalyzing conversion of LPA to phosphatidic acid (PA) or PA to DAG in mammary epithelial cells. Acylation of LPA at the sn-2 position to form PA is catalyzed by members of family of 1-acylglycerol-3-phosphate acyltransferases (AGPAT) (29). Currently, only two members of this family have validated AGPAT activity. Other family members, such as AGPAT6 (see above) and AGPAT8, appear to possess different, or additional, acyltransferase activities (29). As yet, there is no clear indication which members of the AGPAT family catalyze PA formation in the mammary gland. However, high expression levels of GPAT4/AGPAT6 in mammary glands of lactating animals raise the possibility that it may be responsible for both LPA and PA biosynthesis (31). Members of the Lipin family of phosphatidate phosphatases catalyze the conversion of PA to DAG (29). Evidence in cattle, showing that lipin-1 transcripts are relatively abundant in mammary glands of lactating animals, have led to suggestions that this isoform is responsible for the synthesis of DAG used in milk lipid production. However, the importance of lipin-1 in this process has not been validated by loss- or gain-of-function studies.

TAG stabilization

Members of the perilipin (*PLIN*) family of CLD-associated proteins play essential functions in regulating cellular TAG levels (8, 33, 34). Five *PLIN* family members with different functional activities have been identified in eukaryotic cells: *PLIN*1 (perilipin), *PLIN*2 (adipophilin/ADRP), *PLIN*3 (TIP47/mannose-6-phosphate receptor-binding protein 1), *PLIN*4 (S3-12), and *PLIN*5 (OxPAT/lipid storage droplet protein 5) (34–36). A primary function of *PLIN* proteins is the regulation of lipolysis by controlling lipase access to the TAG core of CLD (33, 37–39). Perilipin 2, which appears to be the primary *PLIN* family member expressed by mammary epithelial cells (16, 40–42), is an abundant protein on lipid droplets isolated from the milk of rodents, cattle, and humans (16, 43, 44). PLIN2 transcripts are selectively enriched in mammary epithelial cells in the mouse mammary gland relative to other cell types (42), and their levels undergo about a 30-fold increase during mammary gland differentiation (42). Time course analyses, showing that the pattern of PLIN2 transcript expression in the differentiating mammary gland closely correlates with CLD appearance, growth, and accumulation, implicate PLIN2 as a physiological regulator of CLD production (42). Consistent with this conclusion, PLIN2 deficiency impairs CLD accumulation in mammary epithelial cells of mice without affecting levels of the milk protein β-casein (45). Evidence from mammary epithelial cells of intact mice (45), and from cultured HEK293 cells (39), showing that PLIN2 prevents binding of adipose TAG lipase to CLD suggest that it promotes milk production by stabilizing TAG against lipolysis (Figure 1).

De novo fatty acid synthesis

The fatty acid substrates needed for milk lipid synthesis are derived *de novo* from glucose, or obtained by transfer from the serum (46). Coordinate increases in the activities of enzymes involved in *de novo* fatty acid synthesis (47, 48) and transcript levels of genes encoding these enzymes (49, 50) have been documented in differentiating mammary glands of laboratory animals. Although the exact timing varies between species, induction of these enzymes occurs toward the end of pregnancy or at the beginning of lactation, with expression levels remaining elevated during lactation to maintain milk lipid production (48, 49). The importance of *de novo* fatty acid synthesis in regulating CLD production is suggested by studies of mice lacking protein kinase B (AKT1) (51). AKT1-dependent signaling influences the synthesis of glycerol and lipid from glucose by modulating glucose uptake (3, 51–53) and regulating the activity ATP-citrate lyase, a key regulatory enzyme in *de novo* fatty acid synthesis (54, 55). Mammary epithelial cells in AKT1-null mice exhibit normal morphology and milk protein expression, but their ability to produce CLD is impaired (51). Conversely, in transgenic mice overexpressing constitutively activated AKT1, there is precocious and pronounced accumulation of large CLD in differentiating milk-secreting cells and significant increases in the fat content of milk (3). Collectively, these data argue for a central role for AKT1 in regulating TAG formation in milk-secreting cells by increasing *de novo* glycerol and fatty acid synthesis, and through activation of glucose uptake (Figure 1) (51). These effects of AKT appear to be isozyme specific, as deficiencies in AKT-2 or AKT-3 do not significantly affect mammary gland properties or milk lipid formation, despite altering the metabolic properties of other cell types (51).

Mechanisms of milk lipid secretion

Milk and serum lipids are secreted by distinctly different mechanisms. Serum lipids are secreted by vesicle-mediated exocytosis from hepatocytes or enterocytes as soluble lipoprotein particles (56). Lipoprotein particle synthesis and packaging into secretory vesicles occurs within the ER and Golgi (56–58); thus, serum lipids can be viewed as originating within an external compartment. In contrast, milk lipid secretion occurs by an apocrine-like mechanism, involving apical plasma membrane envelopment of intact CLD, producing membrane bilayer-coated structures known as milk fat globules (MFG) (6, 59). Milk lipids thus remain within the cytoplasmic compartment, and in contact with membrane elements, even after secretion.

Two models have been proposed to explain the process by which CLD undergo envelopment and secretion. In the first, CLD become progressively surrounded by the apical plasma membrane by a process that may be analogous to the budding of viral particles (60, 61). Consistent with this 'budding' model, immunocytochemical evidence indicates that the majority of the MFG membrane envelope is derived from the apical plasma membrane (62). The second model differs from the first in that CLD membrane envelopment is coupled to fusion of peripherally associated secretory vesicles with the apical plasma membrane (59). In this model, the MFG membrane bilayer is hypothesized to be composed of both apical plasma membrane and secretory vesicle membrane elements. Although the weight of current evidence favors the 'budding' model (6), definitive evidence is lacking for either model. Moreover, observations of size and lipid composition differences in MFG (63) raise the possibility that milk lipids may be secreted by more than one mechanism. Nevertheless, a common feature of both models is the interaction of CLD with membrane elements; consequently, identifying the mechanisms mediating such interactions is essential for understanding how milk lipid secretion occurs.

CLD-membrane interactions

CLD are likely to exist in close contact with membrane structures at all times within cells (11, 20, 61, 64), and there is considerable evidence that they form specialized contacts with elements of the apical plasma membrane during milk lipid secretion. In non-lactating animals, CLD appear to be randomly distributed within the cytoplasm of mammary epithelial cells (19). However, once lactation is initiated, they are found predominantly along the inner face of the apical plasma membrane (19, 42). Ultrastructural studies have shown that apical membrane-associated CLD are separated from the membrane bilayer by a 10–20-nm-thick layer of electron dense matrix material (20). Material of similar appearance also separates the lipid droplet surface from the membrane enveloping secreted MFG (20, 65). Matrix material has not been observed in association with CLD in non-secreting cells, nor is it detected on CLD located in other regions of lactating mammary epithelial cells (59), raising the possibility that its formation is secretion dependent, and that it represents structural linkages between CLD and apical membrane elements. Although its composition has not been fully defined, the matrix material associated with membranes surrounding human, bovine, caprine, and mouse MFG are uniquely enriched in three proteins: butyrophilin (BTN1A1/BTN), a receptor glycoprotein member of the immunoglobulin superfamily (66, 67); xanthine oxidoreductase (XOR), a homodimeric purine oxidase that is highly expressed in mammary epithelial cells (68); and PLIN2, which as mentioned earlier, is a CLD-associated protein that is abundantly expressed in mammary epithelial cells and that contributes to CLD production (42, 69). Biochemical, localization, and binding assay evidence indicates that these proteins can form a stable complex with each other, and gene deletion and dominant-negative experiments have shown that each protein is important for milk lipid secretion (70–72). However, the molecular determinants mediating BTN, XOR, and PLIN2 interactions, and the mechanism by which these interactions mediate CLD envelopment and secretion, remain unknown, and alternative mechanisms have been suggested (72, 73). Three models have been proposed to explain how CLD interact with the apical membrane on the basis of structure-function mapping and high-resolution imaging of BTN and PLIN2 (Figure 2).

Tripartite model

This model envisions that CLD-membrane interactions are mediated by PLIN2 on the CLD surface binding to a complex of BTN and XOR located on the cytoplasmic face of the apical plasma membrane (6) (Figure 2). Evidence that BTN and XOR form functionally important linkages is suggested by observations that they are found in constant molar ratios on MFG membranes of cattle throughout lactation and that they can be cross-linked to each other using bifunctional cross-linking reagents (6). Importantly, *in vitro* binding assays showing that purified XOR binds to the C-terminal region (CTR) of BTN with relative high affinity (74) provide direct evidence that BTN and XOR are inherently able to interact with each other to form stable contacts.

Butyrophilin is a type 1 transmembrane protein composed of an externally oriented N-terminal region (NTR) consisting of two Ig folds, and a multidomain C-terminal cytoplasmic region consisting of a stem domain, a B30.2 domain, and a C-terminal cytoplasmic tail (74–76). XOR has been shown to bind stoichiometrically, and selectively, to the B30.2 domain of mouse BTN1A1, the BTN family member expressed in mammary epithelial cells (74). Mutations within the N- or C-terminal portions of B30.2 domain disrupt XOR binding (74), suggesting that binding requires the entire domain and is not mediated by individual submotifs within the domain. B30.2 domains are β-sheet structures containing SPRY and PRY subdomains that are thought to be protein-binding modules. Although structurally similar B30.2 domains are also found in other BTN family members (BTN2 and BTN3), as well as in tripartite motif (TRIM) proteins (76) and the stonefish toxin, stonutoxin (77), to date only the BTN1 B30.2 domain appears to be capable of forming a stable complex with XOR (74). Sequence variability within the protein-binding motifs of B30.2 domain proteins has led to suggestions that individual B30.2 domains may have evolved to recognize specific binding partners. Thus, it is possible that the BTN1A1 B30.2 domain may be a specific XOR binding partner.

Despite the strong *in vitro* binding evidence, the physiological significance of XOR-BTN interactions remains uncertain. CLD interaction with the apical plasma membrane is hypothesized to be initiated by BTN oligomerization, induced by the binding of XOR (6). Data showing that the homodimeric XOR binds two BTN-B30.2 monomers or possibly one BTN-B30.2 dimer (74) support the concept that XOR and BTN can form higher-order structures. However, the C-terminal cytoplasmic region of BTN containing the stem and cytoplasmic tail domains, in addition to the B30.2 domain, is able to dimerize in the absence of XOR, possibly through interactions between

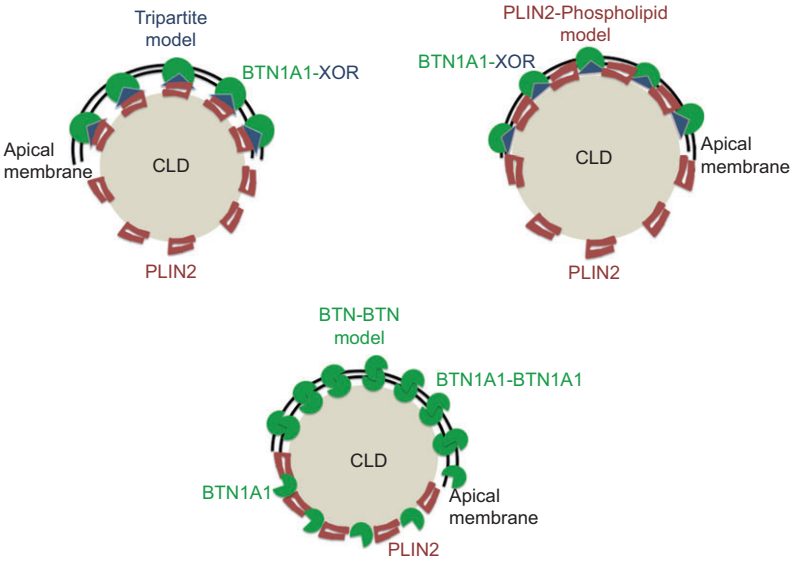

Figure 2 Mechanisms of milk lipid secretion.
Three conceptual models of the mechanisms of milk lipid secretion have been proposed. The tripartite model predicts that CLD associate with the apical plasma membrane through interactions between PLIN2 on the CLD surface and a complex of butyrophilin1A1 (BTN1A1) and XOR on the apical membrane. The BTN1A1-XOR complex is proposed to be formed between a specific B 30.2 domain located within the CTR of BTN1A1 and unknown portions of XOR. PLIN2 is proposed to bind to the BTN1A1-XOR complex through specific domains located within its CTR, and induce clustering of the complex and membrane envelopment of CLD. The BTN-BTN model predicts that homotypic interactions between BTN1A1 molecules located on the CLD surface and the apical plasma membrane leads to the formation of BTN oligomers and membrane envelopment of CLD. The PLIN2-phospholiplid model predicts that interactions between the C-terminal four-helix bundle domain of PLIN2 and the inner phospholipid leaflet of the apical plasma membrane lead to bilayer alterations that induce membrane curvature. Recruitment of the BTN1A1-XOR complex to CLD-membrane interaction sites stabilizes CLD-membrane interactions facilitating CLD membrane envelopment. PLIN2, red U-shaped structures surrounding CLD; BTN1A1, green modified oval structures; XOR, blue triangle structures.

α helical elements within the stem domain (74). Thus, it is possible that both homotypic BTN-BTN interactions and heterotypic XOR-BTN interactions may govern the interactions between BTN and XOR that lead to higher-order structure formation. Significantly, discrete BTN-BTN or BTN-XOR complexes greater than second order have yet to be detected, although heating in SDS induces aggregation of full-length BTN (74). It is unclear whether failure to detect distinct higher-order structures characteristic of BTN-BTN or XOR-BTN oligomers reflects the fact that the correct concentrations and/or conditions required for oligomer formation were not achieved, or that the cytoplasmic domain of BTN is insufficient for oligomerization. Detailed mapping of XOR-BTN and BTN-BTN binding determinants and careful biophysical analyses of these interactions should help resolve conceptual questions related to the role XOR-induced BTN oligomerization plays in milk lipid secretion.

The concept that XOR binds to the apical plasma membrane through its interactions with BTN may also be too simplistic. XOR is found on the limited number of MFG present in milk from BTN-null mice (74); thus, additional, or alternative, mechanisms may be involved in the recruitment of XOR to the apical membrane. Several observations suggest that membrane-associated redox activities contribute to the ability of XOR to bind to the apical membrane. First, the association of XOR with the apical plasma membrane is dynamically regulated by the secretory activity of the mammary gland (78). Second, XOR undergoes reversible thiol oxidation-dependent conversion from a dehydrogenase (XD) to an oxidase (XO) that results in conformation changes that expose a hydrophobic domain (79, 80). Histochemical and biochemical studies indicate that the thiol-oxidized (XO) conformation is enriched in secreted MFG, whereas thiol-reduced XOR is the predominant form of the enzyme in the cytoplasm of mammary epithelial cells (68, 81). Third, apical membrane preparations from lactating mammary tissue (82) and membranes isolated from secreted MFG (68) are reported to contain thiol oxidase activity that converts XD to XO. More recent studies suggest that the flavin-linked sulfhydryl oxidase, quiescin-sulfhydryl oxidase-1 (QSQX1), is responsible for the majority of thiol oxidase activity in bovine milk (83). Members of the quiescin-sulfhydryl oxidase (QSOX) family are known to localize to the ER and the plasma membrane (83), raising the possibility that QSQX1 or a QSOX family member may play a role in XOR oxidation coupled to milk lipid secretion. Whether thiol-oxidized XOR binds to BTN, or to some other membrane component, the presence of BTN nevertheless appears to stabilize XOR membrane interactions, as significantly more XOR is membrane-associated in MFG from wild-type mice than in MFG from BTN-null mice (74). Identifying the enzyme responsible for converting XOR from the XD to XO form is essential for understanding how XOR

binds to the apical membrane and for understanding how this process possibly contributes to milk lipid secretion.

BTN model

Evidence from freeze-fracture replica immunolabeling (FRIL) of isolated MFG has challenged the tripartite model, suggesting instead that BTN-BTN interactions are solely responsible for coupling CLD to the apical plasma membrane (73) (Figure 2). FRIL analysis of the topological distribution of PLIN2, BTN, and XOR on MFG shows that they exhibit divergent localizations that are inconsistent with the concept that they form a tripartite complex. Immunogold-labeled BTN was detected on the cytoplasmic facing surface (P-face) of the phospholipid monolayer surrounding the CLD core, and on the external facing surface (E-face) of the membrane bilayer. BTN labeling on the membrane bilayer E-face localized to a network of ridges that were juxtaposed and spatially matched to the BTN distribution on the P-face of CLD (73). In contrast, immunogold-labeled XOR localized only to the P-face of the CLD monolayer and was diffusely distributed on the monolayer surface, while PLIN2 immunolocalized to the cytoplasmic faces of the membrane bilayer and the CLD monolayer (73). The difficulty in fitting these data to the tripartite model led to the alternative proposal that homotypic interactions between BTN molecules located on the CLD surface and the apical membrane mediate CLD secretion (73). Although this novel model is consistent with functional evidence that BTN is required for CLD secretion (70), it fails to explain how an integral type I membrane glycoprotein such as BTN becomes enriched on the CLD surface; the extensive biochemical evidence documenting that XOR binds to BTN (74, 75, 78); or the *in vivo* evidence showing that disrupting XOR or PLIN2 functions impair CLD secretion (71, 72). Nevertheless, findings showing the presence of a network of ridges enriched in BTN on the membrane bilayer surrounding MFG suggest that BTN interacts to form higher-order structures with specific macromolecular organization. What determines the organization of the BTN ridges and whether such organization reflects the underlying structure of the machinery involved in milk lipid secretion remain to be determined. However, the organization of BTN into spatially distinct regions on the membranes surrounding secreted MFG is consistent with the concept that it can oligomerize, and that its oligomerization may be functionally relevant.

PLIN2 model

PLIN2 is composed of functionally and structurally distinct NTR and CTR (84). The NTR is composed of a PAT domain, which is a region of sequence homology with other PLIN family members that mediates proteasomal degradation (85) and possibly regulates lipase association functions (85, 86), and a putative 11-mer helical repeat region that appears to be responsible for CLD binding (36, 87, 88). The CTR encodes an independently folding four-helix bundle domain with an adjacent hydrophobic motif containing a unique α/β fold (72). This bipartite structural organization makes PLIN2 an ideal candidate to function as an adaptor between CLD and the apical membrane elements involved in milk lipid secretion. Evidence that the PLIN2 CTR plays an important role in regulating milk lipid secretion is suggested by studies in which mutant PLIN2 lacking the CTR was expressed in mammary epithelial cells of lactating mice (72). Mammary epithelial cells expressing this mutant possessed CLD and the mutant protein correctly localized to the CLD surface; however, secretion of CLD coated with the mutant protein was impaired relative to that of CLD coated with exogenously expressed recombinant full-length PLIN2, or with endogenous PLIN2 (72).

Details about the role the PLIN2 CTR plays in milk lipid secretion are limited. However, helix bundle motifs are known to reversibly bind lipids (89), and several lines of evidence indicate that the PLIN2 CTR has membrane-binding functions. First, freeze-fracture studies demonstrated that in addition to being on the CLD surface, PLIN2 is found on the cytoplasmic leaflets of the plasma and ER membranes (11). Second, the PLIN2 CTR localizes to the plasma membrane, but not to CLD, when stably expressed in cultured cells (72), whereas the PLIN2 NTR selectively localizes to the CLD surface (72, 87). Finally, liposome-binding assays demonstrated that the PLIN2 CTR directly binds to phospholipid membranes, through charge-dependent interactions, possibly involving residues within helices 3 and 4 of the four-helix bundle (72). Collectively, these observations indicate that structural features within the PLIN2 CTR direct its membrane-binding functions, and that the PLIN2 CTR binds membranes through direct interactions with phospholipids (Figure 2). These data led to the proposal that interactions between the PLIN2 CTR four-helix bundle domain and the inner phospholipid leaflet of the apical plasma membrane mediate membrane docking of CLD, and possibly initiate the envelopment processes that lead to their secretion (84) (Figure 2).

CLD membrane envelopment

It is unknown how the apical membrane envelops CLD during milk lipid secretion. In other cellular functions involving membrane shape changes, such as endocytosis, vesicle formation, organelle biogenesis, and viral budding, membrane curvature is driven by protein-phospholipid interactions that perturb the bilayer organization and/or by oligomerization of membrane-associated scaffolding proteins that impart bending (90). Although the membrane deformations involved in vesicle formation or viral budding occur on a much smaller scale (nm) than those associated with CLD envelopment (μm), there is evidence that the principles, and in some cases the molecules, governing both sets of processes may be similar. First, CLD may provide a physical template for membrane-bending processes associated with their envelopment. Because CLD in mammary epithelial cells range in diameter from <1 micron to >10 microns in diameter, envelopment mechanisms must be able to accommodate a wide range of

physical dimensions. The different bending geometries and stabilization forces associated with enveloping structures with different sizes suggest that CLD curvature, and the corresponding organization of molecules arrayed on their surface, may provide the appropriate architectural template required to match membrane-bending geometries to CLD of different dimensions. Ultrastructural experiments showing that CLD can be partially, or completely, wrapped by membranes of the ER provide further evidence that CLD can directly induce membrane bending (10). An implication of these findings is that membrane deformation processes associated with CLD envelopment are not specific to the plasma membrane; instead, the ability to bend membrane bilayers may be an inherent property of CLD. Consequently, it is possible that the lipid bilayer perturbations caused by contact with CLD molecules may be the mechanism that initiates their envelopment during milk lipid secretion.

Second, proteins containing helical bundle motifs are known to be capable of deforming and bending membranes (91, 92). The abundance of PLIN2 on CLD in mammary epithelial cells (16), and its ability to bind to membranes (11, 72), possibly through interactions between its C-terminal four-helix bundle motif and membrane phospholipids (72), implicate it in the induction of membrane bending associated with CLD envelopment. In support of this function, Plin3, which has a high degree of genetic and structural similarity to PLIN2 (36), has been shown to induce artificial membrane tubulation (93) and is known to be required for budding of certain types of virus (94). Although the four-helix bundle motifs of Plins 2 and 3 have not been shown directly to mediate membrane bending, the observations that binding of the PLIN2 four-helix bundle domain to membrane phospholipids is mediated by electrostatic interactions is consistent with earlier suggestions that such interactions may be a common feature of proteins that mediate budding in other systems (95).

Third, for many membrane-bending mechanisms, scaffolding proteins are recruited to sites of curvature to help stabilize membrane deformation (90). Lateral interactions between individual BTN molecules and XOR-BTN complexes at the apical membrane are hypothesized to form scaffolding required for CLD envelopment (74). This concept is supported by immunofluorescence and electron microscopic evidence showing enrichment of BTN and XOR on the apical sides of budding CLD in mammary glands of lactating cattle and mice (96, 97), and by immunofluorescence analyses showing that BTN, XOR, and PLIN2 selectively localize near one another at sites of CLD secretion on the apical plasma membrane of lactating mice (78). XOR enrichment on the apical plasma membrane and at sites of CLD secretion appears to be regulated by secretory activity (78), and to depend in part on the presence of BTN (74). These observations implicate recruitment of XOR to sites of CLD-membrane interaction as a possible mechanism regulating assembly and/or stabilization of scaffolding structures involved in CLD envelopment. However, additional studies are needed to define the molecular organization of the XOR-BTN complexes, and to understand how their scaffolding properties are regulated and how they contribute to CLD envelopment.

Summary

Milk lipids are a rich energy source for developing neonates, and mammals have evolved efficient lipid biosynthetic and secretion mechanisms to meet the postnatal growth demands of their young. Although the biochemical steps involved in milk lipid synthesis have been known for decades (98), the identities of the genes mediating these steps and the molecular physiology of milk lipid production have only recently begun to be understood in detail. A central concept derived from studies of transgenic mice is that milk lipid synthesis and secretion appear to play integral roles in mammary gland form and function, and that interference with these processes leads to complete lactation failure (26, 27, 30, 32, 45, 70, 71, 99). Whether the same is true for other species is uncertain, as the metabolic needs and survival of neonatal mice may be particularly dependent on high milk energy content provided by lipids (100, 101). However, gene deletion studies showing that disrupting the synthesis (27, 30) or stabilization of TAG (45) interferes with mammary alveoli maturation during pregnancy suggest that balanced lipid metabolism, particularly those processes involved in milk lipid production, are essential for normal mammary gland development. TAG synthesis precursors, including fatty acids, DAG, PA, and LPA, are known to have cell signaling and gene regulator functions (29). Thus, it is possible that milk lipid synthesis, through the actions of key intermediates, may indirectly contribute to the regulation of mammary gland development.

The mechanism of milk lipid secretion incorporates several features that distinguish it from other apocrine secretion mechanisms that have been described. First, if it is assumed that CLD are components of the cytoplasm, then milk lipid secretion may be the only major secretory pathway that is truly apocrine. Although apocrine secretion processes have been described in other glandular systems, these mechanisms of secretion appear to be hybrids of apocrine and exocrine secretion (102), and the contribution of apocrine processes to the composition of their secretion products is unclear. In contrast, secretion of milk lipids by membrane envelopment of CLD appears to account for the majority, if not all, of the lipid secreted into milk. Second, the intimate association of CLD with the apical membrane during milk lipid secretion appears to distinguish it from other apocrine secretion processes, which are characterized by capture of soluble protein and/or secretory vesicle cargo within membrane blebs without evidence of membrane interaction (102, 103). At present, it is unclear to what extent the cargo influences the formation of these membrane blebs. In contrast, multiple lines of evidence indicate that direct interactions between specific CLD surface proteins and elements of the apical membrane are critical for apocrine secretion of CLD, possibly serving as molecular template to guide this process. Third, rather than being simply lipid storage structures, CLD are now recognized as having organelle-like properties (10, 104, 105). Thus, milk lipid secretion maybe a unique example of apocrine secretion of an organelle.

Why mammary epithelial cells evolved to use a membrane envelopment mechanism for secreting lipids rather than the

ER and Golgi exocrine pathway used for serum lipid secretion is unclear. One possibility is that membrane envelopment of CLD may provide a more efficient pathway for lipid secretion than the ER-Golgi pathway. Although lipid secretion through the ER-Golgi pathway can be significant (serum lipid concentration in humans range from 2% to 10%), milk lipid concentration in many species is well over 10%. In addition, compared with serum lipoprotein particles, MFG have markedly larger diameters (~3–5 μm on average compared with ~0.2 μm for chylomicrons). Consequently, the corresponding volume of lipid contained within a MFG is about 10 000 times that of chylomicron particle, which is consistent with an enormous gain in lipid secretion efficiency that may be required to meet neonatal growth demands.

A second possibility is that MFG have been proposed to play biological functions beyond providing a rich source of energy for neonates (106, 107). In addition to providing structural support, the membrane enveloping MFG is thought to possess bioactive components that potentially contribute to the protection of infants against bacterial infection and possibly their nutrition (107, 108). The concept that MFG are actually secreted organelles also suggests the provocative possibility that MFG are sites of biological reactions important for infant health. It has been known for over 30 years that MFG membranes contain redox enzymes, possibly derived from the ER (65). More recently, proteomic analysis has identified additional enzymes and proteins with possible biological activities on MFG membranes (109) and provided evidence that many of these proteins originate from enveloped CLD (16, 110, 111).

In conclusion, efforts to understand the molecular frameworks regulating lipid synthesis and secretion have led to the identification of critical enzyme and protein determinants of these processes, and are beginning to uncover their underlying structural mechanisms. Importantly, these studies have provided new insight into possible roles lipid metabolism may play in mammary gland development and function, and the potential biological functions of the secreted lipid droplets. However, many important details remain to be learned about the mechanisms controlling CLD biogenesis and secretion, and how these processes are regulated at the physiological level to ensure sufficient milk lipid production during lactation.

Acknowledgements

This study was supported by grants from the National Institutes of Health, 2RO1-HD045962 and PO1-HD38129, to JLM. The author thanks B. Chong, J. Monks, and D. Orlicky for helpful comments and discussions.

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Received June 20, 2012; accepted August 9, 2012