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Proteomics reveal a link between the endoplasmic reticulum and lipid secretory mechanisms in mammary epithelial cells

The synthesis and secretion of lipids by mammary epithelial cells is a highly ordered process that involves several distinct steps. Triacylglycerols are synthesized in the endoplasmic reticulum and incorporated into microlipid droplets which coalesce into cytoplasmic lipid droplets. These are vectorially transported to the apical plasma membrane where they are secreted into the milk surrounded by a membrane bilayer. The origin of this membrane as well as the mechanism by which cytoplasmic lipid droplets form and become surrounded by membrane is poorly understood. Proteomic analysis of the protein composition of milk fat globules and cytoplasmic lipid droplet has revealed that the endoplasmic reticulum is not only involved in the synthesis of the lipid but also potentially contributes to the membrane component of milk fat globules. The proteins identified suggest possible mechanisms of multiple steps during this process. Completion of the proteome of milk fat globule membranes and cytoplasmic lipid droplets will provide the necessary reporter molecules to follow and dissect the mechanisms of the sorting and ultimate secretion of cytoplasmic lipid droplets.

Keywords: Proteomics / Lipid droplet secretion / Milk fat globule membranes / Endoplasmic reticulum / Mass spectrometry EL 4111

1 Introduction

Mammary epithelial cells utilize two distinct pathways for the secretion of milk constituents. Soluble proteins, as well as water, lactose and some minerals, are secreted using the exocytic pathway involving the endoplasmic reticulum (ER), Golgi complex, and secretory vesicles [1]. Lipids, primarily triacylglycerols, are thought to accumulate into microlipid droplets which are released from the ER into the cytosol surrounded by a protein and nonbilayer polar lipid coat [2]. Molecules of this coat have been proposed to play a role in the aggregation of lipid into droplets, the release of the microlipid droplet from the ER, fusion steps into the cytoplasmic lipid droplets (CLDs), and subsequent fusion with the apical plasma membrane [2]. CLDs are secreted from the apical surface of the cell enveloped within a membrane bilayer. To date, the molecular events comprising most of this complex lipid secretory pathway remain unknown.

Lipid secretion in the mammary epithelial cell is unique. The most distinct difference between the mammary epithelial cell and other cell types containing lipid droplets (*e.g.*, hepatocytes, adipocytes) is that mammary CLDs are secreted from the apical surface of the cell surrounded by a membrane bilayer, the milk fat globule membrane (MFGM). Ultrastructural analysis has led to competing hypotheses about the mechanism of secretion and the origin of the membrane bilayer. The most widely accepted view is that CLDs are gradually enveloped by the apical plasma membrane in a budding process [3, 4]. In this hypothesis, the MFGM is derived solely from the apical plasma membrane. A second scenario is that lipid secretion results from the progressive fusion of Golgi-derived secretory vesicles that surround the CLDs within the cytoplasm [5, 6]. This process would lead to a membrane entirely composed of secretory vesicle components. A third view is that secretory vesicles associated with CLDs fuse with each other and the apical membrane [6, 7] to yield a composite membrane derived from both the vesicles and the apical plasma membrane.

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Abbreviations: ADPH, adipophilin; **BTN**, butyrophilin; **CLD**, cytoplasmic lipid droplet; **EM**, electron microscopy; **ER**, endoplasmic reticulum; **ERP**, endoplasmic reticulum protein; **FABP**, fatty acid binding protein; **FAS**, fatty acid synthase; **MFG(M)**, milk fat globule (membrane); **TER**, transitional ER; **WAP**, whey acidic protein; **XDH/XO**, xanthine oxidoreductase

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Another unique feature of lipid secretion in mammary epithelial cells is the vectorial transport of CLDs from the basal region of the cell to the apical membrane for secretion [3]. Microtubules have previously been analyzed and quantitated ultrastructurally in lactating mammary epithelial cells. Most were oriented perpendicular to the apical plasma membrane and concentrated in the apical and medial portions of the cell cytoplasm [8]. These microtubules were found in close contact with secretory vesicles, and treatment with colchicine was found to inhibit exocytosis as well as to suppress milk fat globule secretion [9, 10]. Specific molecules involved in the interaction between the cytoskeleton and secretory vesicles and CLDs remain unknown, and although CLDs are covered by a protein coat, the protein composition is largely undefined.

Only eight of the major protein components of the MFGM have been identified and studied in detail: mucin 1 (MUC-1), xanthine oxidoreductase (XDH/XO), periodic acid Schiff III (PASIII), CD36, butyrophilin (BTN), adipophilin (ADPH), periodic acid Schiff 6/7 (PAS6/7), and fatty acid binding protein (FABP) [11]. Based on biochemical, cytochemical, and developmental studies of the mammary gland and milk fat globules (MFGs), BTN, XDH/XO, and ADPH are proposed to mediate binding of CLDs to the apical plasma membrane [3]. However, there are at least four distinct steps required for the production and secretion of CLDs. (i) Lipids accumulate and are released as microlipid droplets from the ER into the cytoplasm where they coalesce into CLDs. (ii) CLDs are vectorially transported to the apical plasma membrane. (iii) CLDs dock with the apical plasma membrane. (iv) Fusion occurs, resulting in the secretion of CLDs from the apical surface surrounded by a membrane bilayer. Therefore, it is reasonable to assume that there must be multiple other proteins involved in the entire process and that some of these proteins will be present in the MFGM.

Proteomic analysis of cell organelles has been used to identify endogenous proteins and dissect the molecular mechanisms underlying their functions [12–14]. In this study, we present the first installment of the MFGM and the CLD proteomes. 2-D master maps were prepared from MFGMs isolated from milk and CLDs fractionated from mammary gland and liver. Protein spots excised from these gels were identified using reversed-phase LC coupled with tandem mass spectrometry (MS/MS) [15]. Our results suggest that: (i) mammary CLDs differ from liver CLDs in protein composition; (ii) mammary CLDs are intimately associated with membrane-like structures which contain components originating from the ER. (iii) A subset of the proteins present in the MFGM are also present in the mammary CLDs, suggesting that the membranes and adherent proteins associated with the CLDs are involved in the secretory process. Taken together, we propose that the ER may not only contribute the enzymes for lipid synthesis, but also provide some of the molecules required for the vectorial transport and subsequent fusion of the CLD with the apical plasma membrane.

2 Materials and methods

2.1 Isolation of MFGs and MFGMs

Milk was collected from CD1 mice on day 12 of lactation (L12). MFGs were isolated according to Patton and Huston [16]. Briefly, freshly collected warm milk was adjusted to 10% w/v sucrose. Ten volumes of PBS was layered on top of the milk solution and centrifuged at 1500 × *g* for 20 min at room temperature. The floated MFGs were collected with a spatula and resuspended in five volumes of ice-cold PBS. MFGMs were isolated by physical agitation [11]. Briefly, the MFG sample was churned in a Dounce homogenizer until no further clumping of lipid was observed (~ 20 strokes). The suspension was centrifuged at 100 000 × *g* for 1 h at 4°C. Pelleted MFGMs were collected from the bottom of the tube and either washed 1 × in ice-cold PBS by repeating the homogenization and centrifugation step or immediately analyzed.

2.2 Fractionation of CLDs and cytosol

Inguinal mammary glands and livers were removed from the same L12 mice previously milked. The mammary glands were washed in ice-cold PBS to remove the milk present. Both the glands and the livers were minced finely and lightly homogenized using a Polytron PT 10/35 (Brinkmann, Westbury, NY, USA) in 100 mM KH_2PO_4/K_2HPO_4, pH 6.8, 5 mM $MgCl_2$, and 1 μg/mL each of proteolytic inhibitors chymostatin, leupeptin, antipain, and pepstatin. All subsequent sucrose solutions were prepared in this buffer. The homogenate was subjected to low-speed centrifugation (3000 × *g* for 10 min at 4°C) and the resulting postnuclear supernatant (PNS) was loaded in the middle of a sucrose step gradient (1.3 M, 0.86 M, PNS, 0.25 M) and centrifuged at 100 000 × *g* for 1 h at 4°C. The cytosol was collected from the PNS step between the 0.25 M/PNS interface and the PNS/0.86 M interface. The lipid droplets were collected from the top of the gradient and washed 0–3 times as follows. The CLDs were gently resuspended in ten volumes of 0.25 M sucrose in a Dounce homogenizer (~ 5 strokes), floated with centrifugation at 100 000 × *g* for 2 h at 4°C, and recollected from the surface of the solution.

2.3 2-D gel electrophoresis

20–50 μg protein were precipitated using methanol and chloroform [17] for analysis on silver-stained gels. 1–2 mg

protein were precipitated for preparative Coomassie Brilliant Blue R-250 stained gels used for subsequent identification by MS. Precipitated proteins were solubilized in 8 M urea, 4% CHAPS, 1 M thiourea, 83 mM dithioerythritol, 18 mM Trizma base, 0.4% carrier ampholytes pH 3.5–10 (BDH, Poole, UK), and 0.0025% bromophenol blue. Isoelectric focusing was performed using Immobiline dry strips (nonlinear pH range from 3.5–10; Pharmacia, Uppsala, Sweden). The dry strips were rehydrated with the solubilized protein sample by in-gel reswelling [18] and were electrophoresed for 14 h. The second dimension was run on a 9–16% polyacrylamide SDS gel using a Bio-Rad Protean XiII cell (Bio-Rad, Hercules, CA, USA). Silver staining was performed according to SWISS-PROT 2-D PAGE procedures [18]. All gels were scanned using a flatbed scanner, and the resulting images were analyzed by the Melanie II software (Bio-Rad).

2.4 In-gel digestion of 2-D spots

In-gel digests of Coomassie blue-stained 2-D spots were performed as described [19]. Briefly, spots were excised with a scalpel and washed in 100 mM NH_4HCO_3 for 10 min. Proteins were reduced with 3 mM DTT/100 mM NH_4HCO_3 for 20 min at 60°C. After cooling to room temperature, iodoacetamide was added to 6 mM final concentration and incubated in the dark for 20 min at room temperature. The aqueous solution was discarded, and the gel slice was washed in 50% acetonitrile/100 mM NH_4HCO_3 for 20 min. The gel slice was cut into 1 mm^3 pieces and transferred into new 0.5 mL low adhesion Optimum® tubes (Life Science Products, Denver, CD, USA). Gel pieces were dehydrated with the addition of 100% acetonitrile for 15 min. The solvent was removed, and the gel pieces were dried. Gel pieces were reswelled with 0.2 μg modified trypsin (Roche Molecular Biochemicals)/ 25 mM NH_4HCO_3 overnight at 37°C. The supernatant was removed and placed into new 0.5 mL Optimum® tubes. Peptides were extracted from the gel pieces with 100 μL 60% acetonitrile/0.1% TFA for 20 min. The supernatant was removed and added to the previous extract. This extraction was repeated twice. The pooled extract was lyophilized to dryness and reconstituted in 10 μL 5% formic acid immediately before analysis by MS.

2.5 Identification of protein peptides by MS/MS

Peptide samples were centrifuged for 10 min at 15 000 rpm in a microfuge to remove any particulate matter. Samples were then loaded onto a 100 μm fused-silica column with a 5 μm tip. Flow rates were adjusted to ~ 300 nL/min on an HP1100 pump and positioned in front of the heated capillary opening on a Finnigan LCQ (San Jose, CA, USA). Peptides were eluted with an acetonitrile gradient from 98% buffer A/2% buffer B to 40% buffer A/ 60% buffer B where buffer A is 5% acetonitrile/0.5% acetic acid and buffer B is 80% acetonitrile/0.5% acetic acid. Spectra were analyzed using Sequest software [20–22]. Positive identifications were made with the criteria of three separate spectra of high correlation scores. Factors included in the calculation of the correlation score are part of the Sequest software and are discussed elsewhere [22].

2.6 Electron microscopy (EM)

For thin sections, 1 mm^3 tissue pieces were fixed in 2% glutaraldehyde/2% sucrose/100 mM sodium cacodylate, pH 7.35, overnight at 4°C. The tissue was postfixed in 2% OsO_4 in 0.8% potassium ferrocyanide buffered with 100 mM sodium cacodylate, pH 7.35. The tissue blocks were washed with water, dehydrated with increasing EtOH steps (50, 75, 90, 98, 100%), and embedded in Spurr's resin. Sections were post-stained in 5% uranyl acetate in MeOH and Reynold's lead citrate. For negatively stained images, CLDs isolated from mammary epithelial cells and liver were deposited onto formvar-coated copper grids and air-dried. The samples were stained with 2% phosphotungstic acid for 30 s and air-dried again. Samples were imaged using a Phillips CM10 microscope.

3 Results

3.1 Isolation of MFGMs

MFGs were floated from fresh mouse milk collected from lactation day 12 (L12) mice. The MFGMs were separated from the lipid droplet by agitation and subsequently isolated by centrifugation. Analytical (silver-stained) 2-D gels were prepared. Figure 1 shows a comparison between (A) 50 μg total milk and (B) 25 μg MFGM. Major proteins present in both the milk and MFGM samples are boxed. Of the 18 major proteins present in both samples, seven were de-enriched in the isolated MFGMs and later identified by tandem MS/MS as major skim milk or serum proteins, *i.e.*, transferrin, albumin, α-1-protease inhibitor, α-casein, β-casein, ε-casein, and whey acidic protein (WAP). As expected, these proteins were less abundant in the isolated membrane fraction because most of the skim milk proteins would have been separated from the MFGs during the initial float and subsequently diluted away into the supernatant above the membrane pellet during centrifugation [11]. Sixty-five protein spots were enriched in the MFGM and are circled. Clearly, there are a large number of proteins present in the MFGM sample. We have identified some of these proteins using MS/MS and the implications of these identifications will be discussed later in this section.

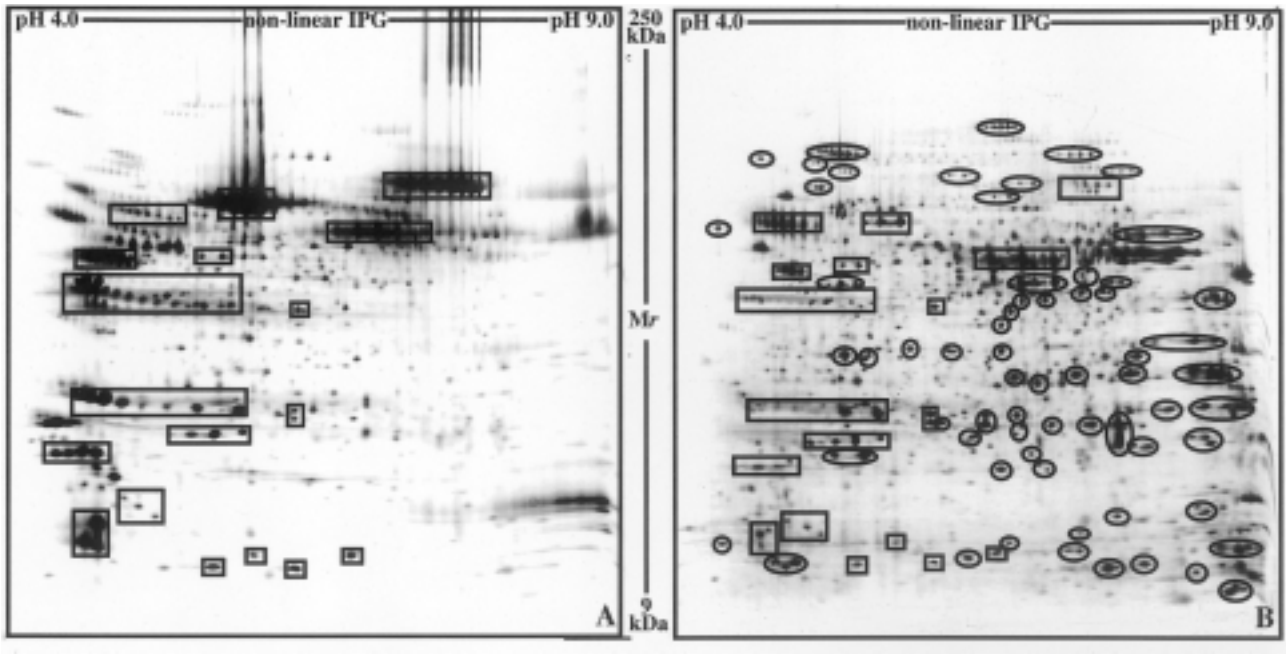

Figure 1. Comparison of the protein composition of total mouse milk and MFGMs by 2-D gel electrophoresis. (A) 50 μg whole milk; (B) 25 μg MFGMs. Silver-stained protein spots present in both samples are boxed. Protein spots enriched in the MFGM sample are circled.

3.2 EM of lactating mammary epithelial cells

Thin sections were prepared from Spurr's embedded lactating mouse mammary glands. The micrographs in Figs. 2A and B show lipid droplets in the apical portion of a mammary epithelial cell. MFGs are present within the alveolar lumen (open star) in Fig. 2A. In addition, a small CLD, almost completely surrounded by membrane, is in the process of being released from the cell surface (closed star). An important observation is that there appears to be very little cytoplasmic component between the lipid droplet and the membrane. The CLD in Fig. 2A (arrow) is observed to be surrounded by vesicles of varying sizes and shapes (large arrowheads). Seen more clearly in a different cell photographed at a slightly higher magnification in Fig. 2B, the vesicle membrane occasionally deforms the surface of the lipid droplet. This observation has been reporeted by others and suggests that there is some physical interaction between the vesicle and the lipid droplet [5]. Frequently, casein micelles are present in the lumen of the surrounding vesicles (small arrowheads). Because casein stains well with osmium, it is visualized using standard EM methods and provides a convenient marker for analysis of secretory compartments. The presence of the casein micelles has supported the hypothesis that these vesicles are Golgi-derived secretory granules. Though there have been no *bona fide* Golgi proteins identified to date, it has been hypothesized that these vesicles are involved in the secretion of CLDs and that the apical plasma membrane is not the sole membrane involved in the secretory process [5].

3.3 Fractionation of mammary and liver CLDs

To investigate the nature of the association between the vesicles and the lipid droplets, CLDs were prepared by cell fractionation from the inguinal mammary glands of the same L12 mice from which the milk was obtained for the MFGM analysis. The isolated CLD samples were negatively stained with phosphotungstic acid and visualized by EM. The micrograph in Fig. 3A shows that the isolated mammary CLDs (arrows) are intimately associated with a variety of flattened membrane-like structures (arrowheads). This sample was washed three times to reduce the presence of membranes that may have bound nonspecifically during the fractionation protocol. There was no apparent change in the amount or appearance of the membranes associated with the CLDs during or after the three washes (unwashed CLDs are not shown).

It was possible that membranes could have remained nonspecifically associated to the CLDs through strong hydrophobic interactions even after multiple washes. Therefore, CLDs were isolated from the livers of the same L12 mice from which the mammary glands and milk were obtained. Liver CLDs are not known to be secreted and

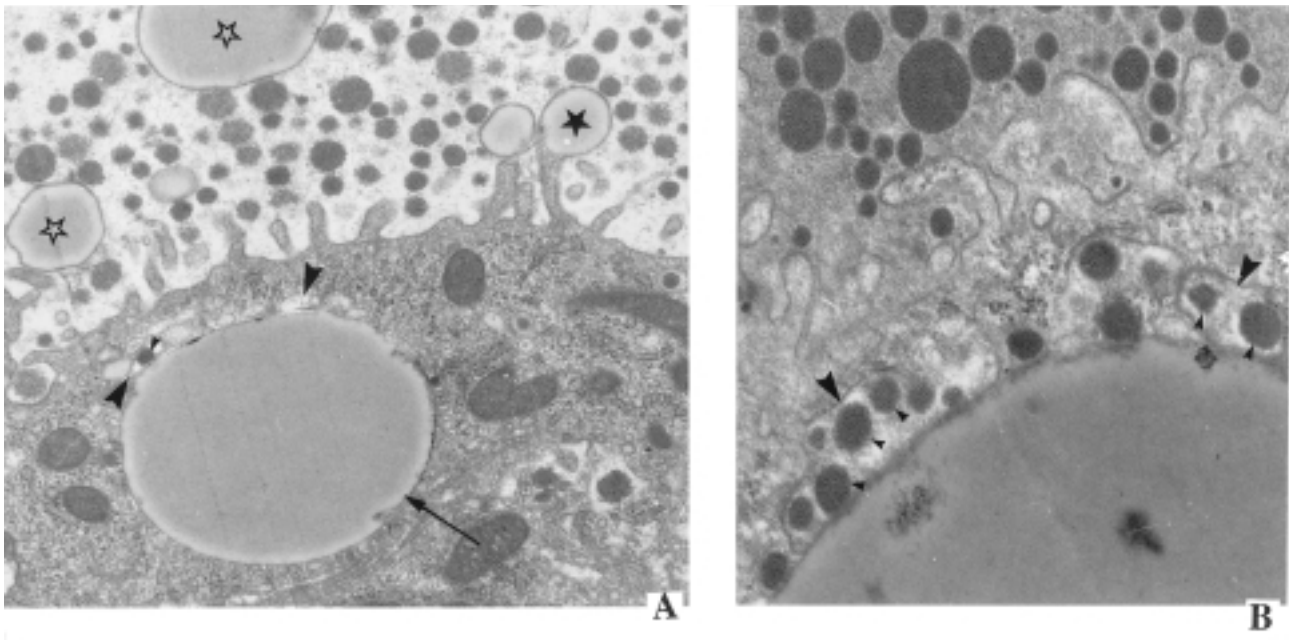

Figure 2. Electron micrographs of CLDs in the apical region of mouse mammary epithelial cells. (A) 600 × magnification; (B) 10 000 × magnification. Symbols: large arrowheads (vesicles associated with the CLDs), small arrowheads (vesicles containing casein micelles), arrow (CLD), open star (secreted MFG), and closed star (CLD in the process of being secreted).

provide an ideal control. If the membranes were associated with the mammary CLDs by nonspecific interactions occurring during the fractionation protocol, then the liver CLDs would be expected to also have membranes bound to their surfaces. Figure 3B shows that there were no detectable membrane structures associated with the unwashed liver lipid droplets isolated in parallel with the mammary CLDs.

3.4 Comparison of mammary and liver CLDs and cytosol

To analyze the protein compositions of the liver and mammary CLDs, analytical (silver-stained) 2-D gels were prepared from both and are shown in Fig. 4B and D, respectively. In addition, 2-D gels were prepared from liver and mammary cytosols to identify major cytosolic proteins (Fig. 4A and C, respectively). Major proteins present in the liver CLDs are boxed (Fig. 4B). These boxed locations are transposed onto the liver cytosol map (Fig. 4A) for comparison. Many of the proteins present in the liver CLDs are either greatly de-enriched or are not at all present in the liver cytosol. Proteins corresponding to the boxed spots in the liver CLDs (Fig. 4B) are also boxed in the mammary CLDs (Fig. 4D). These boxed locations are transposed onto the mammary cytosol map (Fig. 4C) for comparison. Major proteins present in the mammary CLDs but not present in the liver CLDs are circled (Fig. 4D) and the circled locations are transposed onto the mammary cytosol map (Fig.4C) for comparison. As in the liver, many of the proteins present in the mammary CLDs are either greatly de-enriched or are not present at all in the mammary cytosol. The mammary CLDs contain three times more protein spots (84) than the liver CLDs (27) indicating that mammary CLDs are significantly more complex than the liver CLDs. Interestingly, all proteins present in the liver CLDs are also present in the mammary CLDs (boxed spots). These proteins most likely represent some of the basic machinery required for the biogenesis of CLDs. Two of these spots were later identified by MS/MS and found to be ADPH and FABP, two previously characterized MFGM proteins. ADPH has been shown to associate with lipid droplets in many cell types [11], and FABP is thought to function in the transport of fatty acids required for the accretion of lipid droplets in the cytoplasm [11].

3.5 Comparison of MFGM and CLDs by protein identification

An important finding was that many of the spots from the MFGM 2-D map (Figs. 1B, 5A) were identical to the spots on the mammary CLD 2-D map (Figs. 4D, 5B) while there were few matches to the liver CLD 2-D map (Figs. 4B,

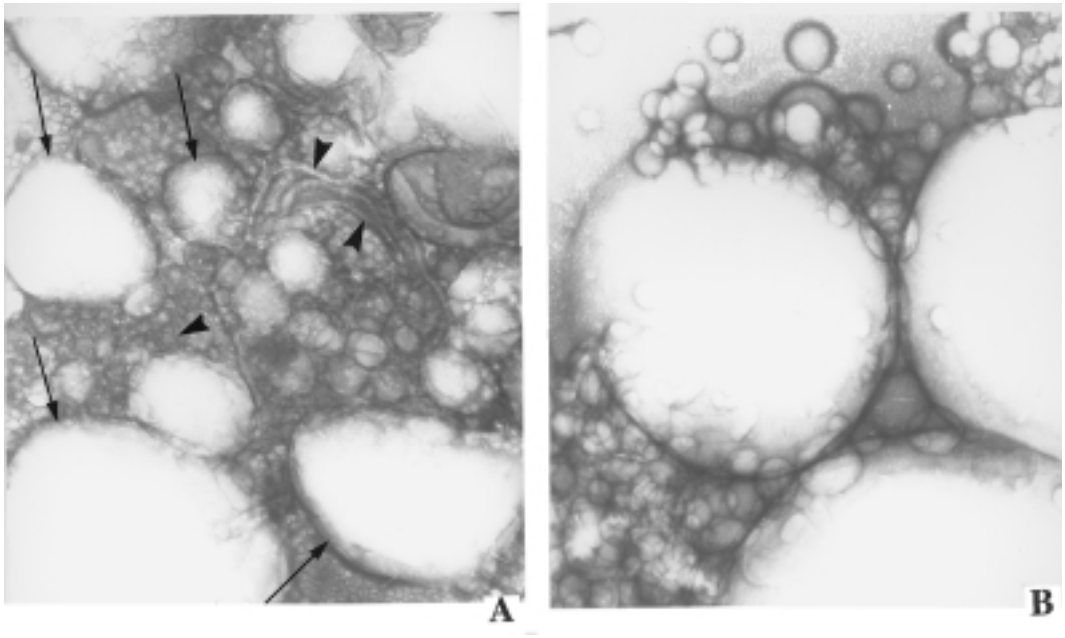

Figure 3. Electron micrographs of negatively stained isolated mouse mammary and liver CLD fractions. (A) Mammary CLDs (washed three times; (19 000 × magnification); (B) liver CLDs (unwashed; 19 000 × magnification). Symbols: arrowheads (membrane-like structures) and arrows (CLDs).

5C). To gain insight into the origin of the membranes surrounding the secreted CLDs, 2-D gel spots were excised from preparative (Coomassie-stained) 2-D gels, and the proteins were identified using reversed-phase LC coupled to MS/MS. Map identification numbers are used to label the spots on these 2-D maps (Figs. 5A, B and C). Protein identifications of the labeled spots are presented in Table 1. All proteins listed in the table were present in the MFGM fraction, and their presence or absence in the mammary and liver CLD fractions is indicated. Of the 29 proteins identified, 19 were also found in the mammary CLDs. Only four were found in the liver CLDs, two of which were the same two major serum proteins (albumin and transferrin) also found in the mammary CLDs. These serum proteins were found in high abundance in the mammary and liver cytosols, presumably due to the rupturing of organelles of the secretory pathway during the homogenization of the tissue and the nonspecific adsorption during the fractionation protocol. The other two proteins found in the liver CLDs were ADPH and FABP, as previously mentioned. An interesting observation was that three isoelectric variants and one truncated form of ADPH were found in MFGMs. However, only the two more basic variants as well as the truncated form were found in mammary CLDs. These same two were found in liver CLDs, but the truncated form was not (Fig. 5, # GM_C). Variable forms of these proteins may be functionally significant.

4 Discussion

4.1 Enrichment of samples analyzed and interpretation of protein identifications

The issue of contamination must be addressed whenever subcellular or secreted fractions are analyzed, especially when using a sensitive technique like 2-D gel electrophoresis coupled to MS/MS. The MFGMs were enriched from the milk expressed by groups of four L12 mice. Multiple mice were used to control for variation among animals. Because the membranes are isolated from milk, there is likely to be little contamination from cytoplasmic lipid droplets or any cellular organelles. The most likely contaminant would be cytosol. Cytoplasmic "crescents" (cytoplasmic inclusions between the lipid droplet and the membrane of the MFG) have been found in varying amounts in the MFGs, depending on the species of animal milked [1]. However, mouse MFGs contain very little cytoplasm, and, in fact, actin is significantly de-enriched in the MFGM fraction compared to the cytosol (data not shown). Nevertheless, contaminants are expected both from milk proteins and cytosol and have been reported by others to be found in their MFGM preparations [11]. In this study, minor amounts of skim milk proteins (α-casein, β-casein, ε-casein, and WAP) and serum proteins (albumin, transferrin, apo A-IV, and apo E/J, fibrinogen γ) were identified in the isolated MFGM fraction.

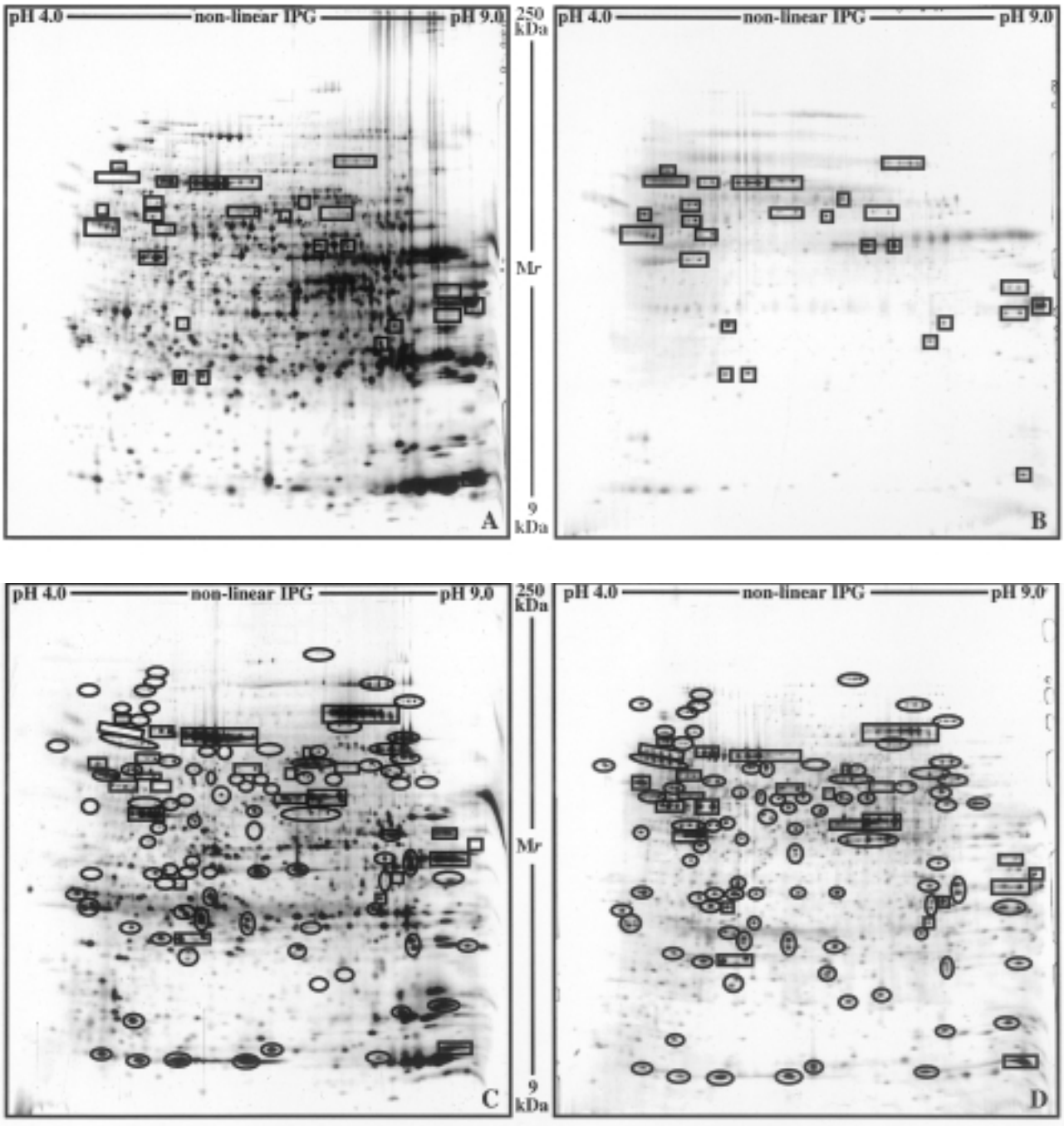

Figure 4. Comparison of the protein composition of mouse mammary and liver CLDs with mouse mammary and liver cytosol by 2-D gel electrophoresis. (A) 40 μg liver cytosol; (B) 20 μg liver CLDs; (C) 40 μg mammary cytosol; (D) 20 μg mammary CLDs. Silver-stained protein spots present in gel (B) are boxed. These boxed locations have been transposed onto gel (A) and gel (C). Silver-stained spots enriched in gel (D) as compared to gel (B) are circled. These circled locations have been transposed onto gel (C).

Mammary CLDs were prepared from the inguinal mammary glands from groups of four L12 mice. The major contaminant to be considered here would be the CLDs from cells other than the mammary epithelial cell, most notably the adipocyte. However, at this stage in development of the mammary gland, most of the cells in the gland are epithelial cells [23]. By immunofluorescence and EM, there are few detectable adipocytes (data not shown). In

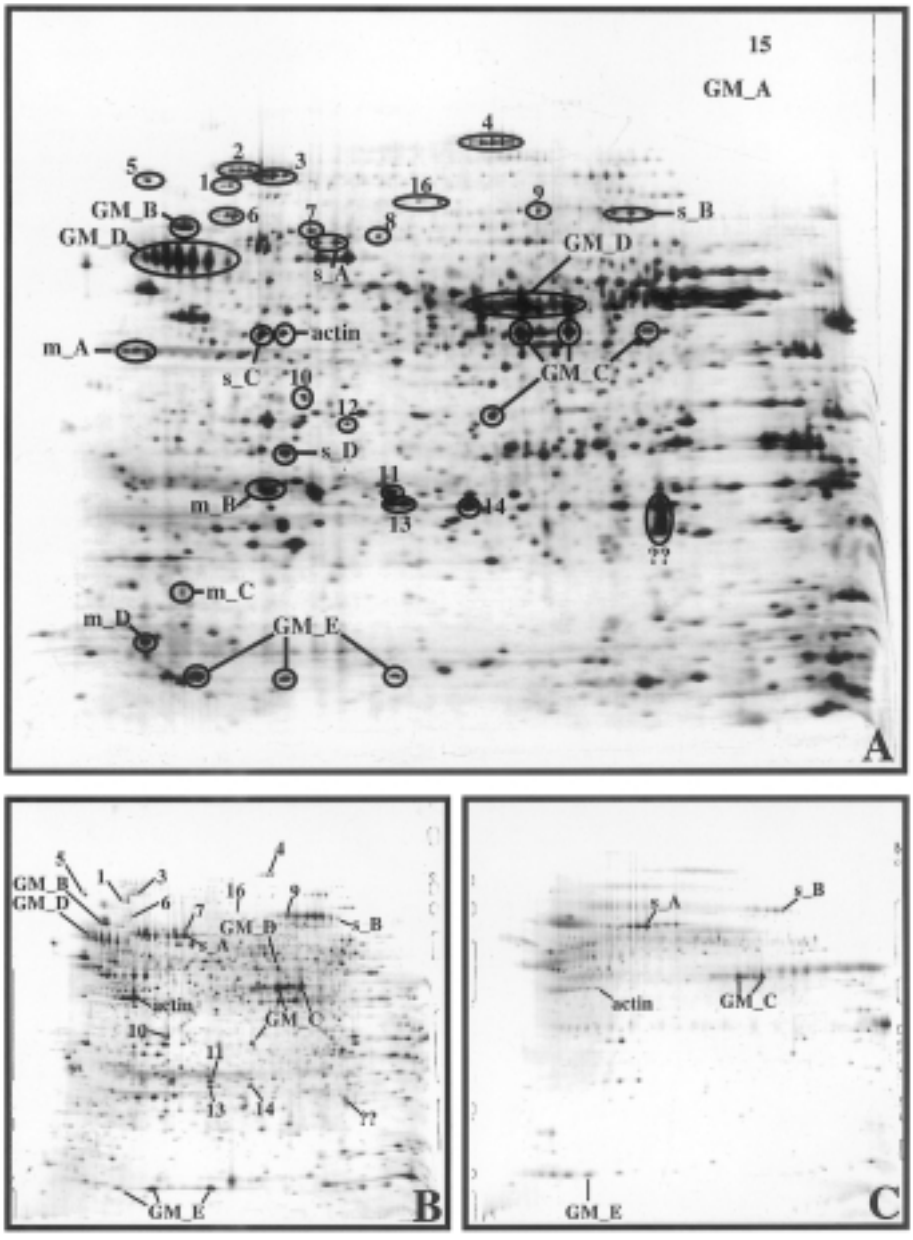

Figure 5. 2-D MFGM master map indicating the locations of the protein spots identified by MS/MS. Mammary and liver CLD maps are included for comparison of identified proteins. Proteins corresponding to # 15 and # GM_A are labeled at the top right corner of the master map. These spots could not be visualized on these 2-D gels. However, they are present in the sample and were identified by MS/MS in bands excised from a 1-D lane run on the edge of the 2-D gel during the second dimension.

the isolation procedure, milk was first washed from the intact mammary glands. The mammary glands were lightly homogenized, followed by the floatation of CLDs on a sucrose step gradient. The isolated CLDs were washed three times, and the fractions were monitored by EM for composition. Importantly, skim milk proteins were not detected in the mammary CLD fraction (Table 1). This implies that the washing of the gland prior to fractionation was adequate. As expected, the major contaminants of this preparation were serum proteins. However, it is clear that many proteins present in the mammary CLDs were not present in the cytosol (circled spots in Fig. 4C and D; Table 1). Liver CLDs were prepared using the same method and, like the mammary CLDs, contained small amounts of serum proteins (boxed spots in Fig. 4A and B; Table 1).

Our controls suggest that proteins identified to be present in both the MFGM and the mammary CLDs are specific and will be discussed in the following sections. The fact that they were isolated and identified from cell homogenates and secreted milk argues strongly that the data are not artifactual. In addition, the liver provides an extremely convincing control. The cytosolic contaminants in the

Table 1 Protein identification by MS[a)]

Map ID#	Identification (abbreviation)	MFGM	Mam. CLD	liver CLD	Mol. mass (kDa)	p*I*
1	TER ATPase	+	+	–	89.3	5.13
2	Fibrinogen γ	+	–	–	99.2	5.57
3	Gephyrin	+	+	–	79.8	5.29
4	Pyruvate carboxylase	+	+	–	129.6	6.57
5	ERP99	+	+	–	91.2	4.91
6	Dynein intermediate chain	+	+	–	68.3	5.16
7	Hsp 70	+	+	–	70.0	6.37
8	Cholesterol esterase	+	–	–	65.8	6.30
9	Motor protein	+	+	–	79.7	6.71
10	TIF32/RPG1	+	+	–	40.1	6.10
11	ER carboxylesterase	+	+	–	30.9	6.31
12	Heterotrimeric G protein β	+	–	–	37.3	6.19
13	ERP29	+	+	–	28.9	6.37
14	Peroxiredoxin IV	+	+	–	28.3	6.50
15	FAS	+	–	–	272.0	5.96
16	Gelsolin	+	+	–	80.8	6.43
MFGM proteins						
GM_A	XDH/XO	+	+[b)]	–[b)]	146.8	8.00
GM_B	BTN	+	+	–	65.0	5.11
GM_C	ADPH	+	+	+	59.2	6.60, 6.75, 6.95
	ADPH (truncated)				40.0	6.53
GM_D	Periodic acid Schiff 6/7 (PAS 6/7)	+	+	–	51.2 65.0	6.55–6.80 4.90–5.50
GM_E	FABP	+	+	+	14.8	5.68, 6.00, 6.31
Milk proteins						
m_A	α-Casein	+	–	–	35.6	4.98
m_B	β-Casein	+	–	–	25.3	5.82
m_C	ε-Casein	+	–	–	16.9	5.26
m_D	WAP	+	–	–	14.7	4.70
Serum proteins						
s_A	Albumin	+	+	+	68.7	6.20
s_B	Transferrin	+	+	+	76.3	6.94
s_C	Apolipoprotein A-IV (ApoA-IV)	+	–	–	49.2	5.86
s_D	Apolipoprotein E/apolipoprotein J (ApoE/ApoJ)	+	–	–	33.2/ 51.5	5.82/ 5.33

a) All protein identifications were by MS/MS unless otherwise indicated.
b) Presence/absence of this protein in the CLD sample was by 1-D immunoblot.

CLD fractions isolated from both tissue types were found to be the same (albumin and transferrin; Table 1), suggesting that proteins present in only mammary CLDs, but not liver CLDs, are specific.

4.2 ER membrane, lumenal proteins and cytosolic proteins may play a role in the formation of the CLDs

The following proteins were identified in both MFGMs and mammary CLDs, but not liver CLDs. These proteins are proposed to play a role in the first step of lipid secretion (Fig. 6).

ERP99 (# 6) is a type I transmembrane glycoprotein enriched in the rough endoplasmic reticulum [24]. The ERP99 oligosaccaride is endoglycosidase H-sensitive, and the glycan was shown by HPLC to be the trimmed form, $Man_8GlcNAc_2$. Therefore, it is presumed to cycle between the ER and the *cis*-Golgi. ERP99 is anchored in the membrane on the *N*-terminal domain, and 75% of the *C*-terminal portion of the protein is exposed on the cytoplasmic face of the ER [25]. From pulse chase experiments in MOPC-315 plasmacytoma cells, ERP99 remained localized to the ER [26]. Synthesis of the protein is upregulated 3- to 10-fold when cells are actively

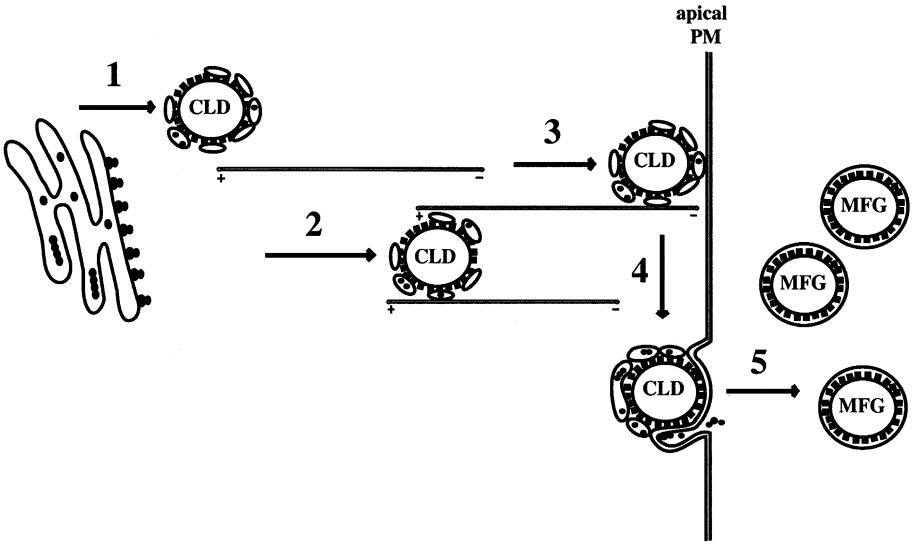

Figure 6. Schematic of five proposed steps involved in lipid droplet secretion in the mammary epithelial cell. (1) Assembly of lipid, protein coat, and vesicles onto the CLDs (2) Attachment of CLDs on to microtubules. (3) Vectorial transport of CLDs to the apical plasma membrane. (4) Fusion events at the apical plasma membrane. (5) Secretion of MFG into the lumen of the alveolus.

secreting [24], suggesting that it may play a role in secretion [27]. ERP99 may be useful as a marker for CLD-associated membranes.

ERP29 (# 13) is a soluble, lumenal ER protein retained in the ER by a variant retention motif, KEEL. ERP29 has homology to protein disulfide isomerase (PDI), but it lacks the characteristic calcium-binding motif of PDI. The expression of ERP29 is upregulated in response to increased secretion but not stress-induced by tunicamycin and calcium ionophores [28]. Therefore, it has been proposed that ERP29 is primarily involved in secretory events [29].

TER ATPase (# 1) is a cytosolic protein (p97) found in a complex with its cofactor p47. It interacts with *N*-ethylmaleimide sensitive fusion protein (NSF) and soluble NSF attachment protein (α-SNAP) to mediate fusion in depletion and add-back cell-free membrane fusion assays using liposomes and microsomes [30]. More recently, p97 was found to regulate the assembly of transitional ER (tER). Depletion of p97 inhibited tER assembly while readdition of purified p97 promoted reconstitution [31].

TIF32/RPG1 (#10) is the 100 kDa subunit (Rpg1p) of the translation initiation factor 3 core complex and is associated with the cytoskeleton. By immunofluorescence microscopy, Rpg1p colocalized with microtubules [32]. In nocodazole-treated samples, the microtubule staining pattern was abrogated. Also, Rpg1p coimmunoprecipitates with α-tubulin as well as intact mibrotubules [32]

Peroxiredoxin IV (# 14) is a new member of a family of enzymes that plays a role in protecting free thiol groups on proteins against oxidative damage [33]. Peroxiredoxin IV (PRxIV) is the secretable form of the enzyme. However, it is largely localized to the ER based on colocalization with calreticulin [33]. PRxIV has a glutathione-dependent peroxidase activity in addition to its thioredoxin-dependent activity and is proposed to play a protective role against oxidative damage by scavenging reactive oxygen species [33].

ER carboxylesterase (# 11) is a resident protein of the ER lumen. In yeast, ER carboxylesterase uses the HDEL retention signal to maintain its localization [34]. It has been shown to be involved in a process leading to the synthesis of luminal triacylglycerols [35].

Hsp70 (# 7) is a member of a family of chaperone proteins that bind to nascent polypeptide chains and partially folded intermediates to prevent their aggregation and misfolding [36].

Pyruvate carboxylase (# 4) is a member of the biotin-dependent carboxylases. It plays a crucial role in gluconeogenesis and lipogenesis by catalyzing the formation of oxaloacetate in response to increased Ca^{2+} levels [37, 38]. It is localized primarily to the cytoplasm [37].

The following two proteins were only found in MFGMs and not in either of the CLD fractions. These will not be discussed further.

Cholesterol esterase (bile salt activated lipase; # 8) is a secreted enzyme involved in the hydrolysis of triglycerides [39]. This enzyme is present in milk and facilitates fat and vitamin absorption and triglyceride hydrolysis in the intestine of the infant [40].

Fatty acid synthase (FAS) (# 15) is a cytosolic protein found to be in a complex with other proteins and lipids in the mammary epithelial cell cytoplasm [41]. This complex was found to contain butyrophilin, xanthine dehydrogenase/oxidase, and a group of small GTP-binding proteins that included ADP-ribosylation factor [41]. This complex interacts with the ER and lipid droplets [41]. FAS was found in MFGMs. FAS is expected to be present in the mammary CLD sample. However, its staining pattern on the 2-D gels (a large faint smear) was not consistent enough to get a positive identification.

A possible mechanism for step 1 (Fig. 6) of lipid secretion is the following. Vesicles enriched with a select group of ER proteins bud from the ER associated with microlipid droplets. Homotypic fusion catalyzed by ER ATPase mediates the fusion of microlipid droplets in the cytoplasm. These fusion events result in the formation of a CLD coated with proteins and large membrane-bound compartments.

4.3 Cytoskeletal components may play a role in vectorial movement of CLDs to the apical plasma membrane

The following proteins were found both in MFGMs and mammary CLDs, but not liver CLDs. These proteins are proposed to play a role in the second and third steps in lipid secretion (Fig. 6).

Dynein intermediate chain (# 6) is the cargo binding component of the minus end-directed microtubule motor complex which plays a role in maintaining the integrity, intracellular location, and function of the Golgi complex [42]. The ATPase which functions as the microtubule motor is localized within the large globular head of the dynein heavy chain, and several classes of light chains are thought to participate in a regulatory role [43]. Traditionally, secretory granules have been thought to utilize the traditional microtubule plus end-targeting motor, the kinesin complex, from the Golgi complex to the cell periphery. However, in the pancreatic acinar cell, targeting of zymogen granules to the apical surface requires an intact microtubule system with the microtubule minus ends close to the apical plasma membrane. Purified zymogen granules were found to be associated with the dynein intermediate and heavy chain but not with kinesin motor components [44]. Immunofluorescence studies showed a zymogen-like distribution for dynein and dynactin in the apical cytoplasm, and a calreticulin staining pattern for kinesin and kinectin in the basal portion of the cell. In addition, secretory granules of nonpolarized chromaffin cells, shown to use the traditional microtubule plus end-targeting motor from the Golgi complex to the cell periphery, were not found to be associated with dynein or dynactin [44].

Motor protein (# 9) is a novel human motor protein found in the heart [45]. To date, its function remains unclear.

Gelsolin (# 16) is a Ca^{2+} and polyphosphoinositide 4,5-bisphosphate (PIP_2)-regulated actin filament severing and capping protein that is implicated in actin remodeling [46]. In response to increased Ca^{2+} and H^+ levels, gelsolin is stimulated to sever actin filaments, after which it remains attached to the barbed end of the filament as a cap. The actin network then remains disassembled because the short actin filaments can no longer attach to one another [47].

Gephyrin (# 3) is a peripheral membrane protein found to be involved in membrane protein-cytoskeletal interactions. Specifically, it binds with high affinity to polymerized microtubules and is thought to anchor inhibitory glycine receptors to the subsynaptic microtubules [48]. Gephyrin interacts with profilin, an actin binding protein that stimulates actin polymerization.

Dynein intermediate chain, motor protein, gelsolin, and gephyrin were all found in both MFGMs and mammary CLDs. The dynein intermediate chain binds membrane vesicles to the heavy chain motor protein, which binds microtubules and functions by hydrolyzing ATP. However, an alternative scenario is that the identified novel motor protein replaces dynein motor protein and functions to link the CLD to the cytoskeleton. The dynein motor is traditionally thought to act as a minus end-directed or “retrograde” motor. As in pancreatic acinar cells, microtubules in the mammary epithelial cell may be oriented with the minus end at the periphery of the cell and the dynein complex would be required for the delivery of cargo to the apical surface. A possible mechanism for steps 2 and 3 (Fig. 6) of the lipid secretion model is the following. The dynein intermediate chain, already associated with its CLD/membrane cargo, bind to one of two possible motors, which moves along the microtubule towards the minus end at the apical plasma membrane. Once at the apical plasma membrane, the actin cytoskeleton is severed by the associated gelsolin to allow for direct contact between the CLDs and the plasma membrane, or “docking”. The gephyrin present may be involved in the initial tethering step. These interactions would complement the interaction between XDH/XO and BTN, which has been proposed to be the “docking” mechanism of CLDs [3]. Alternatively, actin reorganization could occur through the interaction of gephyrin with profilin. Once the initial contact has been made, TER ATPase could mediate further fusion events between vesicles and/or the plasma membrane in step 4 (Fig. 6).

4.4 Release or scission of the MFGs into the lumen of the mammary alveolus

Trimeric G protein β (# 12) is the 37 kDa subunit of a complex composed of three subunits (α, β, γ). Trimeric G proteins are classically known to be involved as modulator or transducer in various transmembrane signaling systems. However, it has also been shown to modulate the exocytosis of secretory granules in prancreatic beta-cells by transient phosphorylation of a histidine residue by a GTP-specific protein kinase [49]. In this study, trimeric G protein β was only found in the MFGMs and not in either CLD

fraction. G-proteins could play a regulatory role in step 5 (Fig. 6) involving the release of the CLDs covered with a membrane composed of proteins originating from ER and plasma membrane, as well as those of other intracellular compartments.

4.5 Conclusion

Using a proteomics approach, we have successfully identified some of the major proteins in MFGMs and mammary CLDs. By performing this type of global analysis, we utilized a nonbiased method for the discovery of specific molecules involved in a unique secretory function. This knowledge provides us with a preliminary set of tools with which to dissect the complexity involved in the secretion of lipids in mammary epithelial cells. Our results provide evidence of the following: (i) mammary CLDs differ from liver CLDs in protein composition; (ii) mammary CLDs are intimately associated with membrane-like structures which contain components originating from the ER; (iii) a subset of the proteins present in MFGMs are also present in mammary CLDs, suggesting that the membranes and adherent proteins associated with CLDs are involved in the secretory process. Functional questions are currently being addressed on a select subset of the proteins identified in this study. Future progress on the MFGM and CLD proteomes will provide the identities of even more molecules involved in the mechanism of lipid secretion, and the ultimate completion of the proteomes will definitively address the specific origin of MFGMs.

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5 References

[1] Mather, I. H., Keenan, T. W., in: Mepham, T. B., (Ed.), *Biochemistry of Lactation*, Elsevier, Amsterdam 1983, pp. 231–283.

[2] Ghosal, D., Shappell, N. W., Keenan, T. W., *Biochim. Biophys. Acta* 1994, *1200*, 175–181.

[3] Mather, I. H., Keenan, T. W., *J. Mam. Gland Biol. Neoplasia* 1998, *3*, 259–273.

[4] Keenan, T. W., Patton, S., in: Jensen, R. G. (Ed.), *Handbook of Milk Composition*, Academic Press, San Diego, CA 1995, pp. 5–49.

[5] Wooding, F. B., *J. Cell Sci.* 1973, *13*, 221–235.

[6] Wooding, F. B., *J. Cell Sci.* 1971, *9*, 805–821.

[7] Kralj, M., Pipan, N., Methka, K., Nada, P., *Biol. Cell* 1992, *76*, 288–293.

[8] Nickerson, S. C., Keenan, T. W., *Cell Tissue Res.* 1979, *202*, 303–312.

[9] Patton, S., *J. Dairy Sci.* 1976, *59*, 1414–1419.

[10] Patton, S., Stemberger, B. H., Knudsen, C. M., *Biochim. Biophys. Acta* 1977, *499*, 404–410.

[11] Mather, I. H., *J. Dairy Sci.* 2000, *83*, 203–247.

[12] Taylor, R. S., Fialka, I., Jones, S. M., Huber, L. A., Howell, K. E., *Electrophoresis* 1997, *18*, 2601–2612.

[13] Rabilloud, T., Kieffer, S., Procaccio, V., Louwagie, M., Courchesne, P. L., Patterson, S. D., Martinez, P., Garin, J., Lunardi, J., *Electrophoresis* 1998, *19*, 1006–1014.

[14] Scianimanico, S., Pasquali, C., Lavoie, J., Huber, L. A., Gorvel, J. P., Desjardins, M., *Electrophoresis* 1997, *18*, 2566–2572.

[15] Yates III, J. R., Morgan, S. F., Gatlin, C. L., Griffin, P. R., Eng, J. K., *Anal. Chem.* 1998, *70*, 3557–3565.

[16] Patton, S., Huston, G. E., *Lipids* 1986, *21*, 170–174.

[17] Wessel, D., Flügge, U. I., *Anal. Biochem.* 1984, *138*, 141–143.

[18] Pasquali, L., Fialka, I., Huber, L. A., *Electrophoresis* 1997, *18*, 2573–2581.

[19] Shevchenko, A., Keller, P., Scheiffele, P., Mann, M., Simons, K., *Electrophoresis* 1997, *18*, 2591–2600.

[20] Gatlin, C. L., Kleemann, G. R., Hays, L. G., Link, A. J., Yates III, J. R., *Anal. Biochem.* 1998, *263*, 93–101.

[21] Dongre, A. R., Eng, J. K., Yates III, J. R., *Trends Biotechnol.* 1997, *15*, 418–425.

[22] Yates III, J. R., Carmack, E., Eng, J. K., in: Celis, J. E. (Ed.), *Cell Biology: A Laboratory Handbook*, Academic Press, San Diego, CA 1998, pp. 539–546.

[23] Hollmann, K. H., in: Larson, B. L., Smith, V. R. (Eds.), *Lactation I*, Academic Press, New York 1974, pp. 3–95.

[24] Lewis, M. J., Mazzarella, R. A., Green, M., *J. Biol. Chem.* 1985, *260*, 3050–3057.

[25] Mazzarella, R. A., Green, M., *J. Biol. Chem.* 1987, *262*, 8875–8883.

[26] Lewis, M. J., Turco, S. J., Green, M., *J. Biol. Chem.* 1985, *260*, 6926–6931.

[27] Koyasu, S., Nishida, E., Miyata, Y., Sakai, H., Yahara, I., *J. Biol. Chem.* 1989, *264*, 15083–15087.

[28] Demmer, J., Zhou, C., Hubbard, M. J., *FEBS Lett.* 1997, *402*, 145–150.

[29] Hubbard, M. J., McHugh, N. J., Carne, D. L., *Eur. J. Biochem.* 2000, *267*, 1945–1957.

[30] Otter-Nilsson, M., Hendriks, R., Pecheur-Huet, E. I., Hoekstra, D., Nilsson, T., *EMBO J.* 1999, *18*, 2074–2083.

[31] Paiement, J., Roy, L., Hendriks, R., Lavoie, C., Gushue, J., Chevet, E., Pelletier, A., Morre, D. J., *ASCB abstract 1739*, 1999.

[32] Hasek, J., Kovarik, P., Valasek, L., Malinska, K., Schneider, J., Kohlwein, S. D., Ruis, H., *Cell Motil. Cytoskel.* 2000, *45*, 235–246.

[33] Okado-Matsumoto, A., Matsumoto, A., Fujii, J., Taniguchi, N., *J. Biochem. (Tokyo)* 2000, *127*, 493–501.

[34] Medda, S., Proia, R. L., *Eur. J. Biochem.* 1992, *206*, 801–806.

[35] Abo-Hashema, K. A., Cake, M. H., Power, G. W., Clarke, D., *J. Biol. Chem.* 1999, *274*, 35577–35582.

[36] Fink, A. L., *Physiol. Rev.* 1999, *79*, 425–449.

[37] Kraus-Friedmann, N., Feng, L., *Metabolism* 1996, *45*, 389–403.

[38] Attwood, P. V., *Int. J. Biochem. Cell Biol.* 1995, *27*, 231–249.

[39] Bruneau, N., Lombardo, D., Bendayan, M., *J. Histochem. Cytochem* 2000, *48*, 267–276.

[40] Mechref, Y., Chen, P., Novotny, M. V., *Glycobiology* 1999, *9*, 227–234.

[41] Keon, B. H., Ankrapp, D. P., Keenan, T. W., *Biochim. Biophys. Acta* 1994, *1215*, 327–336.

[42] Roghi, C., Allan, V. J., *J. Cell. Sci.* 1999, *112*, 4673–4685.

[43] King, S. M., *Biochim. Biophys. Acta* 2000, *1496*, 60–75.

[44] Kraemer, J., Schmitz, F., Drenckhahn, D., *Eur. J. Cell Biol.* 1999, *78*, 265–277.

[45] Icho, T., Ikeda, T., Matsumoto, Y., Hanoaka, F., Kaji, K., Tsuchita, N., *Gene* 1994, *144*, 301–306.

[46] Sun, H. Q., Yamamoto, M., Mejillano, M., Yin, H. L., *J. Biol. Chem.* 1999, *274*, 33179–33182.

[47] Cooper, J. A., Schafer, D. A., *Curr. Opin. Cell Biol.* 2000, *12*, 97–103.

[48] Prior, P., Scmitt, B., Grenningloh, G., Pribilla, I., Multhaup, G., Beyreuther, K., Maulet, Y., Werner, P., Langosch, D., Kirsch, J., *Neuron* 1992, *8*, 1161–1170.

[49] Kowluru, A., Seavey, S. E., Rhodes, C. J., Metz, S. A., *Biochem. J.* 1996, *313*, 97–107.