The protein and lipid composition of the membrane of milk fat globules depends on their size

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ABSTRACT

In bovine milk, fat globules (MFG) have a heterogeneous size distribution with diameters ranging from 0.1 to 15 μm . Although efforts have been made to explain differences in lipid composition, little is known about the protein composition of MFG membranes (MFGM) in different sizes of MFG. In this study, protein and lipid analyses were combined to study MFG formation and secretion. Two different sized MFG fractions ($7.6 \pm 0.9 \mu\text{m}$ and $3.3 \pm 1.2 \mu\text{m}$) were obtained by centrifugation. The protein composition of MFGM in the large and small MFG fractions was compared using mass-spectrometry-based proteomics techniques. The lipid composition and fatty acid composition of MFG was determined using HPLC-evaporative light-scattering detector and gas chromatography, respectively. Two frequently studied proteins in lipid droplet biogenesis, perilipin-2 and TIP47, were increased in the large and small MFG fractions, respectively. In the large MFG fraction, besides perilipin-2, cytoplasmic vesicle proteins (heat shock proteins, 14–3–3 proteins, and Rabs), microfilaments and intermediate filament-related proteins (actin and vimentin), host defense proteins (cathelicidins), and phosphatidylinositol were higher in concentration. On the other hand, cholesterol synthesis enzymes [lanosterol synthase and sterol-4- α -carboxylate 3-dehydrogenase (decarboxylating)], cholesterol, unsaturated fatty acids, and phosphatidylethanolamine were, besides TIP47, higher in concentration in the small MFG fraction. These results suggest that vesicle proteins, microfilaments and intermediate filaments, cholesterol, and specific phospholipids play an important role in lipid droplet growth, secretion, or both. The observations from this study clearly demon-

strated the difference in protein and lipid composition between small and large MFG fractions. Studying the role of these components in more detail in future experiments may lead to a better understanding of fat globule formation and secretion.

Key words: milk, fat globule, membrane proteome, phospholipids, triglycerides

INTRODUCTION

Milk fat is secreted by the mammary gland in the form of globules. These milk fat globules (MFG) are composed of a lipid core (mainly triglycerides, **Tg**) covered by a 3-layer membrane structure called the milk fat globule membrane (MFGM, mainly phospholipids and proteins). In bovine milk, the diameter of the fat globules varies from ~ 0.1 to $\sim 15 \mu\text{m}$. The composition of fatty acids and phospholipids varies between different sized MFG. For example, smaller MFG contained a higher amount of PUFA (Lopez and Menard, 2011; Mesilati-Stahy et al., 2011). It was also shown that the phospholipid composition of the MFGM varied between different MFG sizes (Cohen et al., 2015; Mesilati-Stahy et al., 2015). Although the differences in lipid composition have been studied in different sized MFG, little is known on the relation between MFG size and protein composition of the MFGM. The study of MFG size and composition has several industrial implications. For example, cheese characteristics (Michalski et al., 2003), as well as MFG coalescence (Lopez et al., 2010), were shown to be related to the protein and lipid composition of different sized MFG. In addition, different lipids have different metabolic fates in the human body (Michas et al., 2014), and differences in lipid composition depending on MFG size thus also have nutritional implications.

In the last few decades, improvements in the understanding of milk component synthesis and secretion were achieved (McManaman and Neville, 2003; Neill,

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2006). In the mammary gland, milk Tg are synthesized between the layers of the endoplasmic reticulum (**ER**) membrane and secreted into the cytoplasm as micro lipid droplets (**LD**). Part of these micro LD fuse to form large LD when moving to the apical plasma membrane. Via a special budding process, the LD are released from epithelial cells to milk as MFG. These MFG are covered by a 3-layer membrane structure (MFGM) that is derived from the outer leaflet of the ER and the apical plasma membrane of the cell. The MFGM is composed of phospholipids and proteins. Of the proteins in MFGM, butyrophilin, xanthine dehydrogenase, and perilipin-2 (**ADFP**) were shown to be involved in the budding process of MFG to milk (Mather and Keenan, 1998; Heid and Keenan, 2005; Dewettinck et al., 2008). The secretion of both smaller and larger LD results in the wide range of MFG sizes as found in milk. The mechanism that regulates formation and secretion of different sized LD from the mammary gland is, however, not well elucidated (Mather and Keenan, 1998).

Lipid droplets of different sizes can be found also in adipose (Hörl et al., 2011) and liver (Stringer et al., 2010) cells. Two main factors on the surface of LD, phospholipids and proteins, were suggested to control LD size (Cohen et al., 2015). For example, in murine adipocytes, the level of the **PAT** (perilipin/adipophilin/adipose differentiation related proteins/TIP47) protein ADFP decreased simultaneously with a reduction in intracellular LD size upon dietary supplementation of *trans*-10,*cis*-12 CLA (Stringer et al., 2010). Enzymes increasing Tg synthesis or inducing Tg hydrolysis have also been suggested to regulate LD size (Thiam et al., 2013). The protein activity on the LD surface was hypothesized to be changed by interactions with specific phospholipids (Walther and Farese, 2009). Phospholipids in the membrane were indeed shown to affect stability and size of LD in the cytoplasm. For example, phosphatidylethanolamine (**PE**) was found to destabilize the LD membrane and thereby induce LD fusion, resulting in larger LD (Thiam et al., 2013). Phosphatidylcholine (**PC**), on the other hand, has been suggested to stabilize the LD surface and hence inhibit fusion, resulting in smaller droplets in *Drosophila* (Guo et al., 2008). Whether these mechanisms are also involved in the modulation of lipid droplet size in the mammary epithelial cells has yet to be determined.

The abundance of these proteins and lipids within the mammary epithelial cell may influence the size of the MFG. Therefore, a detailed proteome and lipidome analysis of different sized MFG fractions may reveal which cellular components play a role in size regulation of the MFG during the synthesis and secretion process. Hence, MFG of individual cows were separated accord-

ing to their size, resulting in a fraction of small MFG and a fraction of large MFG. The protein and lipid composition of both fractions was analyzed by mass spectrometry-based proteomics for protein composition, and HPLC-evaporating light scattering detector (**ELSD**) and GC for lipid composition. The comprehensive analysis of proteins and lipids in different MFG size fractions may provide targets for further studies aimed at understanding what determines MFG size.

MATERIALS AND METHODS

Milk Samples

Milk samples were collected from 7 healthy Holstein cows in mid lactation (100–200 d) between December 2011 and January 2012 from the Wageningen University Farm (Wageningen, the Netherlands). Raw milk samples were obtained immediately after milking in the morning and transported to the laboratory for subsequent analyses on the same day. Milk composition was analyzed using a MilkoScan 134A/B (Foss Electric, Hillerød, Denmark).

Milk Fat Globule Separation

Milk samples were heated to 40°C in a water bath. The heated milk samples were then centrifuged at $190 \times g$ for 10 min at 15°C to separate cream and skim milk. The top layer of cream was subsequently removed for centrifugation with PBS (pH 6.8, 0.1 M, 1:10, vol:vol) at $390 \times g$ for 10 min at 10°C. The washing solution was discarded after centrifugation. This step was repeated 3 times. The cream obtained using this procedure was the large MFG fraction. The skim milk was centrifuged at $390 \times g$ for 10 min at 10°C. This step was repeated 3 times. The cream after each centrifugation step was discarded except after the last step. The obtained cream was washed 3 times with PBS (pH 6.8, 0.1 M, 1:10, vol:vol) at $390 \times g$ for 10 min at 10°C. The cream obtained using this procedure was the small MFG fraction.

Determination of Globule Diameters

The small and large MFG fractions were diluted with 35 mM EDTA/NaOH buffer (pH 7.0, 1:1, vol:vol) to dissociate casein micelles, followed by heating to 40°C. The globule diameters were analyzed using a Mastersizer 2000 (Malvern Instrument Ltd., Malvern, UK). The refractive index of the cream samples was 1.458 at 633 nm. Milli-Q water was used as a dispersant, and its refractive index was 1.33.

Separation of Proteins from Milk Fat Globules

A 0.4% SDS solution was added to the small and large MFG fractions (1:1, vol:vol). After cooling at 4°C for 15 min, the cream solution was sonicated for 3 min (Transsonic 700, Singen, Germany). It was then centrifuged at $1,500 \times g$ for 5 min at 10°C. The bottom part of the solution contains the proteins. Because serum proteins have been washed away during the initial MFG separation, this protein fraction is assumed to represent the MFGM proteins. Protein concentration was determined using BCA assay (Thermo Scientific Pierce BCA protein assay kit, Waltham, MA).

Proteomics Analysis by Filter-Aided Sample Preparation-Dimethyl Labeling-nanoLC-Orbitrap-Tandem MS

The MFGM proteins (10 µg) of the large and small MFG fraction were prepared and compared by using filter-aided sample preparation (FASP)-dimethyl labeling-nanoLC-Orbitrap-MS/MS. The procedure as described below was identical to that from Lu et al. (2011).

Filter-Aided Sample Preparation. The protein clean up and digestion method used was based on FASP with some modifications. Our procedure was as follows: protein samples were diluted in the SDT-lysis buffer (100 mM Tris/HCl pH 8.0 + 4% SDS + 0.1 M dithiothreitol) to get a 1 µg/µL protein solution. The samples were then incubated at 95°C for 5 min and cooled down to room temperature. Ten microliters of each sample was directly added to a Pall 3K omega filter and centrifuged at $20,000 \times g$ for 1 min at room temperature. Then, 100 µL of UT (100 mM Tris/HCl pH 8.0 + 8 M urea) was added to the filter and centrifuged at $20,000 \times g$ for 30 min. Next, 100 µL of IAA (0.05 M iodoacetamide in UT) was added and mixed, followed by 10 min of incubation at room temperature and centrifugation at $20,000 \times g$ for 30 min. Subsequently, 110 µL of UT was added to the filter after which it was centrifuged at $20,000 \times g$ for 30 min at room temperature. This step was repeated 2 times with 120 and 130 µL of UT, respectively. Then, 140 µL of 0.05 M NH_4HCO_3 (ABC) was added to the filter unit, after which it was centrifuged at $20,000 \times g$ for 30 min at room temperature. Finally, 100 µL of ABC containing 0.5 µg of trypsin was added, followed by overnight incubation at room temperature. Finally, the filter unit was centrifuged at $20,000 \times g$ for 30 min. The sample obtained was acidified with 10% trifluoroacetic acid to adjust the pH of the sample to 2 to 4. These samples were ready for either direct nanoLC-LTQ-Orbitrap-tandem MS (MS/MS) analysis or dimethyl labeling.

Dimethyl Labeling. The peptide samples prepared with FASP were labeled as described before (Boersema et al., 2009). C18+ Stage tip columns were made in house. The C18+ Stage tip column was washed 3 times with 200 µL of methanol. It was then conditioned with 100 µL of 1 mL/L of HCOOH. The peptide samples were loaded on the C18+ Stage tip column followed by washing with 100 µL of 1 mL/L of HCOOH. The column was then slowly flushed with 100 µL of the labeling reagent (light: CH_2O or heavy: CD_2O) in about 10 min. The column was washed with 200 µL of 1 mL/L of HCOOH. The labeled peptides were eluted with 50 µL of 70% acetonitrile/30% 1 mL/L of HCOOH from the columns. Two samples, one with light dimethyl label and one heavy dimethyl label, were then mixed, after which the volume was adjusted to exactly 100 µL by adding 1 mL/L of HCOOH. These samples were ready for analysis by nanoLC-LTQ-Orbitrap-MS/MS.

NanoLC-LTQ-Orbitrap-MS/MS. The samples were analyzed by injecting 18 µL of sample (Proxeon nLC) over a 0.10×32 mm Prontosil 300–5-C18H (Bischoff, Germany) pre-concentration column (prepared in house) at a maximum pressure of 270 bar. Peptides were eluted from the pre-concentration column onto a 0.10×200 mm Prontosil 300–3-C18H analytical column with an acetonitrile gradient at a flow rate of 0.5 µL/min. The gradient consisted of an increase from 9 to 34% acetonitrile in water with 1 mL/L of HCOOH in 200 min followed by a fast increase in the percentage acetonitrile to 80% (with 20% water and 1 mL/L of HCOOH in both the acetonitrile and the water) in 3 min as a column cleaning step.

Between the pre-concentration and analytical column, an electrospray potential of 3.5 kV was applied directly to the eluent via a solid 0.5-mm platinum electrode fitted into a P777 Upchurch microcross. Full scan positive mode FTMS spectra were obtained between m/z 380 and 1,400 on a LTQ-Orbitrap XL (Thermo Electron, San Jose, CA). The MS/MS scans of the 10 most abundant doubly and triply charged peaks in the FTMS scan were recorded in data dependent mode in the linear trap (MS/MS threshold = 5,000, 60 s exclusion duration).

Identification and Quantification of Proteins. In total, 7 LC-MS/MS raw files were generated (the large vs. small MFG fractions of milk samples from 7 cows). Each run with all MS/MS spectra obtained was analyzed using MaxQuant 1.2.2.5 with Andromeda as the peptide search engine (Cox and Mann, 2008; Cox et al., 2011). The database for peptide/protein searches was a concatenated bovine reference database downloaded from Uniprot (www.uniprot.org, 25–10–15) with reverse sequences generated by MaxQuant. The contaminants database of MaxQuant was also used for

peptide/protein searches, which includes sequences of trypsin and human keratins. Identification and quantification of proteins was simultaneously performed by MaxQuant. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine, N-terminal acetylation, and deamidation of asparagine or glutamine were set as variable modifications for both identification and quantification. Peptide tolerance was set at 10 ppm, and fragment ions tolerance at 0.5 amu. Mass deviation of 0.5 Da was set as the maximum allowed for MS/MS peaks, and a maximum of 2 missed cleavages were allowed. Maximum false discovery rates were set to 1%, both on the peptide and protein level. Minimum required peptide length was 6 AA for both identification and quantification. A minimum of 2 peptides for each protein was required for identification. Dimethyl labeling was based on doublets with dimeth-Lys0 and dimethNter0 as light, and dimethLys4 and dimethNter4 as heavy. Only razor and unique peptides were used for quantification. For each LC-MS run, MaxQuant calculated normalized peptide ratios so that the median of their logarithms was zero, which corrects for unequal protein loading, assuming that the majority of proteins show no differential regulation, as described by Cox and Mann (2008). The median H/L normalized ratio for each protein was used, and the average H/L normalized ratios of 7 biological replicates were used for statistical analysis.

Proteomics Data Analysis and GO Enrichment Analysis

One sample *t*-tests were performed on the protein ratio of each protein in 7 comparisons (large MFG/small MFG fraction), comparing the average log₂ ratio to 0. The Gene Ontology (GO) enrichment analysis of significantly changed proteins ($P < 0.05$) was done using DAVID bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov/>; Huang et al., 2009). The GO terms shown in the Results section were significantly enriched compared with the GO terms of all proteins identified in this study. Significance was defined as $P < 0.05$ where the Fisher Exact test was adopted to measure the gene enrichment in GO terms.

Lipid Extraction

For lipid extraction, analytical reagent-grade methanol and chloroform were purchased from Bio-Lab Ltd. Laboratories (Jerusalem, Israel). For HPLC analysis, dichloromethane, methanol, and ethanol, HPLC-grade and analytical reagent-grade, were purchased from Bio-Lab. The Tg standard triolein (>99% pure) was purchased from Supelco (Bellefonte, PA). Cholesterol

(>99% pure) and phospholipid standards were supplied by Sigma-Aldrich Israel Ltd. (Rehovot, Israel), and consisted of PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, purity 99%), phosphatidylinositol (PI; L- α phosphatidylinositol ammonium salt, from bovine liver, purity 98%), phosphatidylserine (PS; 1,2-dioleoyl-sn-glycerol-3-phospho-L-serine sodium salt, purity 95%), phosphatidylcholine (PC; 1,2-dioleoyl-sn-glycero-3-phosphocholine, purity 99%), and sphingomyelin (SM; from bovine brain, purity 97%). As an internal standard for free fatty acids, C11:0 (undecanoic acid, purity 99%) from Sigma-Aldrich was used.

A protocol adapted from the cold extraction procedure developed previously (Folch et al., 1957) was used for the extraction of total lipids from the milk. Total lipids were extracted from samples containing approximately 200 mg of cream using chloroform-methanol (2:1, vol/vol) as described previously (Mesilati-Stahy et al., 2012). For the HPLC analysis, 100 μ L of chloroform-ethanol (97:3 vol/vol) was added to the evaporated tubes containing lipids and stored at -20°C until injection into the HPLC.

Phospholipid and Cholesterol Analysis

Quantification of PL and Tg, and determination of lipid class, were performed by HPLC (HP 1200, Agilent Technologies, Santa Clara, CA) combined with an evaporative light-scattering detector (ELSD1200, Agilent Technologies). The separation process was managed by ChemStation software (Agilent Technologies), which permitted the acquisition of data from the ELSD, with an injection volume of 10 μ L. The separation was conducted as described previously (Argov-Argaman et al., 2012) using normal-phase chromatography on a silica column (Zorbax, Agilent Technologies). Calibration, and lipid concentration and composition, were determined using external standards (Sigma-Aldrich).

The PL were identified and quantified by normal-phase liquid chromatography (HP 1200, Agilent Technologies) combined with an ELSD (1200 series ELSD, Agilent Technologies). The method employed for lipid separation, which uses dichloromethane, methanol, and double-distilled water, was as previously described (Mesilati-Stahy et al., 2011). Briefly, a column (Zorbax RX-SIL, 4.6×250 mm, Agilent Technologies) was heated to 50°C , and flow was set to 1 mL/min. The ELSD was heated to 65°C , nitrogen pressure was 3.9 bar, a no. 5 filter was used, and gain (sensitivity) was set to 7 for the first 11 min and then changed to 9 until the end of the run to enable detection of lower-abundance lipid components. Injection volume was 20 μ L. This allowed separation of triacylglycerol, 2 isomers of diacylglycerol, monoacylglycerol, cholesterol,

Table 1. Size parameters of fat globules in whole milk, the small milk fat globule (SMFG), and the large milk fat globule (LMFG) fraction, and milk composition of the whole milk¹

Fraction	d ₄₃ , μm	d ₃₂ , μm	Specific surface area, m ² /g of fat	Protein content per unit area, mg/m ²	Fat, %	Protein, %	Lactose, %
Whole milk	3.95 ± 0.26 ^a	3.47 ± 0.27 ^a	1.89 ± 0.15 ^a	3.56 ± 0.45 ^a	3.95 ± 0.56	3.58 ± 0.23	4.53 ± 0.07
SMFG	3.32 ± 1.21 ^b	2.93 ± 1.06 ^b	2.23 ± 0.81 ^b	3.38 ± 0.89 ^a			
LMFG	7.61 ± 0.90 ^c	4.31 ± 0.17 ^c	1.16 ± 0.05 ^c	4.06 ± 0.20 ^a			

^{a-c}Denotes significant differences when paired sample Student *t*-test was applied (*P* < 0.01).

¹Diameters $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$; $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of fat globules with diameter d_i ; specific surface area = $6/(d_{32} \times \varphi)$ assuming $\varphi = 0.92$ g/L for fat globule (Walstra et al., 2005). Data values represents mean ± SD of milk samples and separated MFG fractions from seven cows

free fatty acids, PE, PI, PS, PC, and SM. Quantification was based on areas under the standard curve of each lipid standard. The power equations were Tg, $y = 0.0014x^{0.8695}$ ($R^2 = 0.995$); cholesterol, $y = 0.0245x^{0.581}$ ($R^2 = 0.9925$); PE, $y = 0.1369x^{0.437}$ ($R^2 = 0.9908$); PI, $y = 0.0103x^{0.7918}$ ($R^2 = 0.9898$); PS = $1.73x^{0.41}$ ($R^2 = 0.99$); PC, $y = 0.0408x^{0.5077}$ ($R^2 = 0.9986$); and SM, $y = 0.0667x^{0.5287}$ ($R^2 = 0.9981$). The weight of each of the polar lipids in 1 mL of milk was calculated according to the power equations, and its weight percentage of total membrane lipids (PE + PI + PC + SM + cholesterol) was determined.

Fatty Acid Composition

Milk-fat composition was measured at the Qlip laboratory (Zutphen, the Netherlands). Milk fat was extracted from the milk samples, and FAME were prepared from fat fractions as described in ISO Standard 15884 (ISO-IDF, 2002a). Methyl esters were analyzed according to ISO Standard 15885 (ISO-IDF, 2002b) on a Trace GC Ultra chromatograph (Thermo Electron Corporation), using a Varian Fame Select column (100 m × 0.25 mm ID; Varian Inc., Palo Alto, CA). The initial temperature was held at 70°C for 1 min, raised to 225°C at 3°C/min, and held at 225°C for 5 min. A volume of 1 μL was injected. Each peak was identified and quantified using pure methyl esters (Sigma-Aldrich, Zwijndrecht, the Netherlands; Larodan, Malmö, Sweden). The fatty acids were expressed as a proportion of total fat weight.

RESULTS

Fat Globule Size Distribution

Two fractions containing MFG with an average diameter of 3.3 ± 1.2 μm and 7.6 ± 0.9 μm, were separated from milk samples of 7 individual cows (Table 1). As expected, the small MFG fraction had a larger specific membrane surface area than the large MFG fraction (Table 1).

Fatty Acid Composition of Different Sized MFG

The fatty acid composition of the 2 MFG size fractions was determined by GC. Table 2 gives an overview of the significantly changed fatty acids between the 2 different globule sizes. This table shows that the significant differences were in unsaturated, medium, and long-chain fatty acids. Of the SFA, only the long-chain C17:0, C18:0, and C20:0 differed significantly.

The total amount of saturated and unsaturated fatty acids and CLA were compared (Table 3). This table shows that the amount of SFA was significantly higher in the large MFG fraction, whereas that of UFA (total UFA, total MUFA, and CLA) was higher in the small MFG fraction.

Phospholipid Composition in Different Sized MFG

The phospholipid composition of the different sized MFG fractions was determined using HPLC-ELSD (Table 4). A significant difference was found in the

Table 2. Significant different fatty acids (g/100 g of fat) in the large and small milk fat globule (MFG) fractions¹

Fatty acid, g/100 g of fat	C-10:1	C-12:1	C-14:1 <i>cis</i> -9	C-17:0	C-18:0	C-18:1 <i>trans</i> -10	C-18:1 <i>cis</i> -9	C-18:3 <i>cis</i> -9,12,15	C-18:2 <i>cis</i> -9, <i>trans</i> -11 (CLA)	C-20:0
Small MFG	0.348	0.123	1.324	0.474	8.879	0.190	17.530	0.396	0.383	0.120
Large MFG	0.324	0.112	1.232	0.483	9.463	0.195	17.071	0.386	0.358	0.127

¹Significant increases are shown in bold.

Table 3. Total amount of fatty acids and cholesterol (g/100 g of fat), by type, in the large and small milk fat globule (MFG) fractions¹

Fatty acid, g/100 g of fat	Cholesterol	Total SFA	Total UFA	CLA
Small MFG	0.294	71.691	28.309	0.383
Large MFG	0.217	72.452	27.548	0.358

¹Significant increases are shown in bold.

content of PI and PE. No difference was observed in the other 3 phospholipids.

Protein Composition of Different Sized MFG

In total, 4,688 peptides were identified and quantified, resulting in 156 proteins identified and quantified that were used for further statistical analysis. Forty-nine and 23 proteins were significantly higher in concentration in the large and small MFG fractions, respectively (Tables 5 and 6). Next, a functional analysis of significantly changed proteins was performed based on GO. In the large MFG fraction, these proteins were mainly associated with protein transport/stabilization, host defense, and cytoskeleton organization. These proteins showed enrichment in the subcellular locations “cytoplasmic vesicle” and “intracellular non-membrane-bounded organelle,” which was mainly cytoskeleton/chromosome (Figures 1 and 3). Proteins significantly enriched in the small MFG fraction were mainly associated with lipid metabolic processes and cholesterol synthesis and showed “apical membrane” and “integral to membrane” as enriched subcellular location (Figures 2 and 3). Not only the low abundant proteins, but also the major proteins differed between the large and small MFG fractions, with an increase of lactadherin (Q95114, **MFGE8**), perfilin-2 (Q9TUM6, **ADFP**), and glycosylation-dependent cell adhesion molecule 1 (P80195, **GLYCAM1/PP3**) in the large MFG fraction and an increase of platelet glycoprotein 4 (P26201, **CD36**) and mucins (Q8WML4, **MUC1**; Q8MI01, **MUC15**; and F1MRA6, **MUC16**) in the small MFG fraction. The most significant difference between the large and small MFG fractions was in the host defense proteins.

Cathelicidins (P22226, **CATHL1**, P54228, **CATHL6**, and P33046, **CATHL4**), lactoferrin (P24627, **LTF**), complement C3 (Q2UVX4, **C3**), lymphocyte cytosolic protein 1 (Q3ZC00, **LCP1**), and monocyte differentiation antigen CD14 (Q95122, **CD14**) were more abundant in the large MFG fraction. There was also a different distribution of lipid synthesis enzymes between the MFG fractions. Fatty acid synthase (Q71SP7, **FASN**) was enriched in the large MFG fraction, while acyl-CoA synthetase (Q1LZF6, F1MEX9, **ACSL**), lanosterol synthase (P84466, **LSS**), acetyl-CoA carboxylase 1 (Q9TTS3, **ACACA**), sterol-4- α -carboxylate 3-dehydrogenase (decarboxylating; Q3ZBE9, **NSDHL**), and glycerol-3-phosphate acyltransferase 4 (A3FPG8, **AGPAT6**) were enriched in the small MFG fraction.

DISCUSSION

Unlike factors that may influence the size of intracellular LD in other cell types, factors determining the size of secreted MFG have not been extensively studied. Due to the contribution of MFGM proteins and lipids in MFG synthesis and secretion, we performed a comprehensive compositional analysis of different sized MFG fractions to find proteins and lipids that may influence MFG size and thereby provide targets for further studies in this area.

The fatty acid composition as reported in Tables 2 and 3 showed that the large MFG fraction was characterized by a lower concentration of UFA and a higher concentration of stearic acid compared with the small MFG fraction. This is in agreement with previous literature on the difference in fatty acid composition between large and small MFG (Timmen and Patton,

Table 4. Phospholipid composition (weight % of total phospholipids) in the large and small milk fat globule (MFG) fraction¹

Phospholipid weight, %	PI*	PE*	PS	PC	SM
Small MFG	4.79	27.46	7.77	37.18	22.80
Large MFG	10.30	19.50	8.04	36.46	25.71

¹PI = phosphatidylinositol; PE = phosphatidylethanolamine; PS = phosphatidylserine; PC = phosphatidylcholine; SM = sphingomyelin.*Significant at $P < 0.05$.

Table 5. Proteins with significantly higher concentration in the large milk fat globules (MFG) fraction and their average log₂ protein ratio (large/small) in the 7 individual cows¹

Uniprot entry	Gene name	Protein name	Ratio	RA, %	GOBP
Q95114	MFGE8	Lactadherin	0.50 ± 0.15	41.57	Angiogenesis, phagocytosis, cell adhesion
Q9TUM6	ADFP	Perilipin-2	0.12 ± 0.12	33.84	Long-chain fatty acid transport, lipid storage
P80195	GLYCAM1	Glycosylation-dependent cell adhesion molecule 1	0.65 ± 0.39	4.52	
F1N726	GP2	Glycoprotein 2 (Zymogen granule membrane)	0.71 ± 0.75	4.49	
P60712	ACTB	Actin, cytoplasmic 1	2.35 ± 1.18	3.4	Filament, lipid transport, lipid droplet secretion, protein folding
Q0IIG8	RAB18	Ras-related protein Rab-18	0.33 ± 0.23	2.77	Regulation or transcription, protein transport, small GTPase mediated signal transduction
P22226	CATHL1	Cathelicidin-1	4.25 ± 1.47	2.01	Defense response
P62803		Histone H4	4.23 ± 1.57	1.3	Nucleosome assembly
Q71SP7	FASN	Fatty acid synthase	0.79 ± 0.63	1.26	Lipid biosynthesis
P46193	ANXA1	Annexin A1	3.11 ± 0.99	1.15	Cell cycle, signal transduction, positive regulation of vesicle fusion, cell proliferation, arachidonic acid secretion
Q3T101	IGL@	IGL@	0.78 ± 0.63	1.01	Defense response
P24627	LTF	Lactoferrin	2.20 ± 1.77	0.82	Ion transport, defense response
P28782	S100A8	Protein S100-A8	3.55 ± 0.99	0.79	Chemotaxis
F1N2R1	STOM	STOM protein	0.50 ± 0.46	0.63	
P48616	VIM	Vimentin	3.55 ± 1.27	0.61	Intermediate filament-based process
P19120	HSPA8	Heat shock cognate 71 kDa protein	0.65 ± 0.50	0.6	Regulation of transcription, protein folding, response to stress, regulation of cell cycle
P54228	CATHL6	Cathelicidin-6	3.51 ± 1.07	0.49	Defense response
A7MAZ5	HIST1H1C	Histone H1.3	3.98 ± 1.39	0.44	Nucleosome assembly
Q8SPP7	PGLYRP1	Peptidoglycan recognition protein 1	4.61 ± 1.92	0.41	Peptidoglycan catabolic process
P0CH28	UBC	Polyubiquitin-C	0.53 ± 0.34	0.41	
P33046	CATHL4	Cathelicidin-4	4.19 ± 1.36	0.35	Defense response
F1MH40	IGK	IGK protein	1.12 ± 0.72	0.34	
Q2UVX4	C3	Complement C3	2.51 ± 0.70	0.3	Defense response
P68103	EEF1A1	Elongation factor 1-α 1	0.90 ± 0.65	0.26	GTP catabolic process, translation
Q05927	NT5E	5-nucleotidase	0.44 ± 0.40	0.24	Nucleotide catabolic response, negative regulation of inflammatory response
P62261	YWHAЕ	14-3-3 protein epsilon	0.67 ± 0.69	0.23	Protein targeting, negative regulation of protein dephosphorylation
Q9XSJ4	ENO1	Alpha-enolase	2.39 ± 1.66	0.23	Glycolysis
P04272	ANXA2	Annexin A2	0.53 ± 0.54	0.21	Angiogenesis, fibril organization, positive regulation of vesicle fusion
P10096	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	2.85 ± 1.44	0.2	Glycolysis
Q5E9F7	CFL1	Cofilin 1 (nonmuscle)	1.43 ± 1.42	0.18	Cytokinesis, protein phosphorylation, cytoskeleton organization, positive regulation of actin filament depolymerization
Q3ZC00	LCP1	Lymphocyte cytosolic protein 1 (L-plastin)	3.24 ± 1.23	0.17	Immune response, intracellular protein transport, actin filament bundle assembly
F1N514	CD5L	CD5L protein	0.61 ± 0.57	0.16	
Q3T149	HSPB1	Heat shock 27 kDa protein 1	2.51 ± 1.12	0.15	Response to stress
Q95122	CD14	Monocyte differentiation antigen CD14	0.39 ± 0.35	0.14	Defense response
P63103	YWHAZ	14-3-3 protein zeta/delta	1.20 ± 0.90	0.14	Protein targeting
Q76LV1	HSP90AA1	Heat shock protein HSP 90-α	1.38 ± 0.94	0.13	Protein folding, response to stress, negative regulation of proteasomal ubiquitin-dependent protein catabolic process
Q148J4	RAB2A	Ras-related protein Rab-2A	0.19 ± 0.13	0.11	Small GTPase mediated signal transduction, protein transport
P62157	CALM	Calmodulin	1.27 ± 1.16	0.09	Calcium binding and release
P05307	P4HB	P4HB protein	1.18 ± 0.89	0.08	Glycerol ether metabolic process, cell redox homeostasis
Q0VCX2	HSPA5	78 kDa glucose-regulated protein	1.40 ± 0.86	0.08	
P68250	YWHAB	14-3-3 protein β/α	1.13 ± 0.90	0.07	Protein targeting, negative regulation of protein dephosphorylation
Q5E9B1	LDHB	L-Lactate dehydrogenase B chain	1.99 ± 1.14	0.07	Glycolysis

Continued

Table 5 (Continued). Proteins with significantly higher concentration in the large milk fat globules (MFG) fraction and their average log₂ protein ratio (large/small) in the 7 individual cows¹

Uniprot entry	Gene name	Protein name	Ratio	RA, %	GOBP
A1L528	RAB1A	Ras-related protein Rab-1A	0.27 ± 0.29	0.06	Small GTPase mediated signal transduction, protein transport
Q27965	HSPA1B	Heat shock 70 kDa protein 1B	0.83 ± 0.52	0.06	DNA repair, response to stress, anti-apoptosis
P19803	ARHGDIA	Rho GDP-dissociation inhibitor 1	0.65 ± 0.48	0.05	Rho protein signal transduction, positive regulation of GTPase activity
P49951	CLTC	Clathrin heavy chain 1	1.74 ± 0.85	0.05	Vesicle mediated transport/protein transport
P38657	PDIA3	PDIA3 protein	1.71 ± 1.11	0.05	Glycerol ether metabolic process, positive regulation of apoptosis, cell redox homeostasis
P19483	ATP5A1	ATP synthase subunit α	2.08 ± 0.85	0.04	Cell proliferation, ATP metabolic and proton transport, lipid metabolic process
Q863B3	SND1	Staphylococcal nuclease domain-containing protein 1	0.55 ± 0.44	0.02	Regulation of transcription

¹Proteins were listed in order of descending protein intensity. Ratio is presented as average ± SD. RA = relative abundance (relative to butyrophilin as %). GOBP = Gene Ontology biological process.

Table 6. Proteins with significantly higher concentration in the small milk fat globules (MFG) fraction and their average log₂ protein ratio (large/small) in the 7 individual cows¹

Uniprot entry	Gene name	Protein name	Ratio	RA, %	GOBP
Q27960	SLC34A2	Sodium-dependent phosphate transport protein 2B	-0.51 ± 0.21	2.71	Ion, phosphate transport
P02663	CSN1S2	Alpha-S2-casein	-1.47 ± 1.06	1.96	Transport, host defense
P26201	CD36	Platelet glycoprotein 4	-0.43 ± 0.25	1.84	Transport, cell adhesion
Q1LZF6	ACSL1	Acyl-CoA synthetase long-chain family member 1	-0.54 ± 0.16	1.57	Lipid metabolic process
Q8WML4	MUC1	Mucin-1	-0.31 ± 0.32	1.01	DNA damage response, negative regulation of transcription
Q2KIS4	DHRS1	Dehydrogenase/reductase (SDR family) member 1	-0.42 ± 0.29	0.79	Oxidation-reduction process
Q8MI01	MUC15	Mucin 15, cell surface associated	-0.61 ± 0.30	0.67	
P84466	LSS	Lanosterol synthase	-0.32 ± 0.23	0.51	Cholesterol biosynthetic process
G3N2D8	GGT1	Gamma-glutamyltranspeptidase 1	-0.55 ± 0.20	0.5	Glutathione metabolic process
Q2T9M8	SNAP23	Synaptosomal-associated protein	-0.20 ± 0.17	0.43	Exocytosis
Q3SYS6	CHP	Calcium-binding protein p22	-0.39 ± 0.14	0.29	Protein export from nucleus, negative regulation of protein phosphorylation, cytoplasmic microtubule organization
P15396	ENPP3	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	-0.51 ± 0.13	0.27	Phosphate metabolic process, immune response
Q9TTS3	ACACA	Acetyl-CoA carboxylase 1	-0.47 ± 0.21	0.23	Lipid metabolic process
F1MD24	SGLT1	Na ⁺ /glucose cotransporter	-0.18 ± 0.15	0.23	Glucose transport
Q3SX32	TIP47/M6PRBP1	Mannose 6 phosphate receptor binding protein 1/TIP47	-1.19 ± 0.43	0.2	
Q3ZBE9	NSDHL	Sterol-4- α -carboxylate 3-dehydrogenase, decarboxylating	-0.48 ± 0.37	0.17	Cholesterol biosynthetic process
D5HSX1	PRKG2	cGMP-dependent type II protein kinase	-0.10 ± 0.08	0.13	Protein phosphorylation
G3N0S9		Uncharacterized protein	-1.21 ± 0.63	0.13	
F1MRA6	MUC16	Mucin-16	-0.45 ± 0.18	0.11	
A5PK13	LRRC8C	Leucine-rich repeat-containing protein 8C	-0.31 ± 0.31	0.11	
A4FUD2	HSD17B7	17- β hydroxysteroid dehydrogenase	-0.33 ± 0.19	0.11	Cholesterol biosynthetic process
F1MEX9	ACSL3	Long-chain-fatty-acid-CoA ligase 3	-0.24 ± 0.16	0.08	Lipid metabolic process
A3FPG8	AGPAT6	Glycerol-3-phosphate acyltransferase 4	-0.26 ± 0.23	0.06	Lipid metabolic process

¹Proteins were listed in order of descending protein intensity. Ratio is presented as average ± SD. RA = relative abundance (relative to butyrophilin as %). GOBP = Gene Ontology biological process.

Figure 1. Biological functions of proteins with significantly higher concentration in the large MFG fraction compared with the small MFG fraction. Functions were assigned based on gene ontology as well as literature references for each protein. The number of proteins in each function category is labeled in the chart.

1988; Briard et al., 2003). It has been hypothesized that this difference in lipid composition might be related to ease of fusion of droplets with different fatty acid composition (Timmen and Patton, 1988). An alternative cause of this difference might be the increased amount of MFGM material, with the small MFG fraction having twice the amount of membrane as the large MFG fraction (Table 1). As MFGM is relatively rich in UFA

(Jensen and Nielsen, 1996), the higher amount of UFA in the small MFG fraction may be related to this relative increase in membrane. Briard et al. (2003) have calculated that there should be up to 1% more oleic acid (C18:1 *cis*-9) in small MFG than in large MFG, if the compositional differences between globule sizes would be due to their MFGM content only. This may therefore explain the increased UFA content, oleic acid

Figure 2. Biological functions of proteins with significantly higher concentration in the small MFG fraction compared with the large MFG fraction. Functions were assigned based on gene ontology as well as literatures references for each protein. The number of proteins in each function category is labeled in the chart.

Figure 3. Gene ontology (cellular component) enrichment of significantly changed proteins in the large and small MFG fractions by using DAVID Bioinformatics Resources 6.7. The GO terms shown in this figure were significantly enriched ($P < 0.05$) compared with the GO of all proteins identified in this study. Black = GO term enriched in the large MFG fraction. Grey = GO term enriched in the small MFG fraction.

content specifically, in the small MFG fraction (Tables 2 and 3).

Of the phospholipids, PE is significantly different (Table 4). The concentration of PE in the membrane is significantly lower in the large MFG compared with the small MFG fraction. Although the function of this polar lipid within mammary gland epithelial cells is not well understood, the function of PE has been implied in other biological systems, as described in the Introduction. The PE was previously found to destabilize the LD membrane, resulting in LD fusion and an increase in size (Thiam et al., 2013). This may thereby explain the lower concentration of PE in the large MFG fraction. The size-dependent fatty acid and phospholipid composition suggests a different polarity of the large and small MFG fractions resulting from length and unsaturation differences in the fatty acids and polarity and structure differences in the phospholipids in each MFG size group. Such polarity differences could in turn change the affinity of the MFGM for proteins. This may explain, in part, the proteome differences between the large and small MFG fractions.

A previous study on the proteome of different sized globules in milk compared lactosomes (nanometer-sized and Tg depleted lipid-protein assemblies) to MFG. The number of proteins that was significantly increased between the large and small MFG fractions in our study (49 and 23, respectively), is very similar to what was found previously when comparing the proteome of regular MFG with that of lactosomes (Argov-Argaman et al., 2010). However, lactosomes are much smaller than the small MFG fraction in our study, and also do not carry a Tg core, so a further comparison of the proteome with their study was not done.

The PAT proteins, which are among the most abundant proteins in the MFGM, have been considered essential in lipid biogenesis (Ducharme and Bickel, 2008; Thiele and Spandl, 2008; Walther and Farese, 2009). In

this study, ADFP and TIP47, 2 members of the PAT family of proteins, were identified in bovine MFGM. Interestingly, the abundance of these 2 proteins varied in different MFG size fractions. The ADFP preferentially localized in the large MFG fraction, whereas TIP47 localized in the small MFG fraction, which is consistent with a previous study (Wolins et al., 2005). The reason for this difference in distribution is, however, not clear. Accumulation of neutral lipids, fusion of small LD, or inhibition of Tg lipolysis have been suggested to play a role in creating or maintaining large LD (Ducharme and Bickel, 2008; Thiele and Spandl, 2008; Walther and Farese, 2009). The ADFP promoted the formation of large LD by inhibiting lipolysis in mammary epithelial cells (Russell et al., 2007), which thereby may explain the higher concentration of ADFP in the large MFG fraction. The concentration of GLYCAM1/PP3, another protein that could inhibit lipolysis (Sorensen et al., 1997), was also higher in the large MFG fraction (Table 5).

In the present study, a higher concentration of cytoplasmic vesicle proteins was observed in the large MFG fraction, which suggests a stronger association of cytoplasmic vesicle proteins with the membrane of large MFG/LD. Three groups of cytoplasmic vesicle proteins are present in higher concentration in the large MFG fraction (Table 5), including heat shock proteins (P19120, **HSPA8**; Q3T149, **HSPB1**; Q76LV1, **HSP90AA1**; Q0VCX2, **HSPA5**; and Q27965, **HSPA1B**), 14-3-3 proteins (P62261, **YWHAE**; P63103, **YWHAZ**; and P68250, **YWHAB**), and proteins from the Rab GTPase family (Q0IIG8, **Rab 18**; Q148J4, **Rab 2A**; and A1L528, **Rab 1A**). Two reasons for this difference are possible. First, larger LD may attract more membrane-bound vesicles on their surfaces because of the long-range London-Van der Waals and electrostatic forces (Wooding, 1971; Wu et al., 2000). Second, heat shock proteins, 14-3-3 proteins, and Rab

GTPases, which have been previously reported in bovine and human MFGM (Reinhardt and Lippolis, 2006; Cavaletto et al., 2008), may play a role in stabilization of large MFG. Several heat shock proteins function as intracellular chaperones for other proteins. They play an important role in protein-protein interactions, such as folding and assisting in the establishment of proper protein conformation, and prevention of unwanted protein aggregation. The HSP70 (Q27965, **HSPA1B**) has been observed in intracellular LD in rat adipocytes where its suggested functions were stabilization of the membrane monolayer, transporting of nascent proteins to LD, and refolding of denatured proteins in the membrane layer (Jiang et al., 2007). Rab proteins are a group of proteins that play an important role in membrane trafficking, including vesicle formation, vesicle transportation, and membrane fusion (Mountjoy et al., 2008). Among the Rab proteins, Rab18 was observed in the surface of intracellular LD in HepG2 cells where it was involved in the apposition of ER and LD for transport of neutral lipid/fatty acids from ER to LD resulting in the growth of LD (Ozeki et al., 2005). Based on this existing knowledge of the function of these cytoplasmic vesicle proteins on growth of LD and stability of its membrane, we propose that the increase of these proteins in large MFG may increase intracellular stabilization of the LD membrane.

The enrichment in the large MFG fraction of non-membrane-bound proteins, mainly cytoskeleton proteins (vimentin, plasmin L, and actin), as well as cytoskeleton-associated proteins (annexins and cofilin), suggests the involvement of these proteins in the heterogeneity in MFG size. The cytoskeleton, mainly microtubules, has previously been proposed to function in LD transport and fusion in a range of cell types, including epithelial cells of the mammary gland (Wu et al., 2000; Bostrom et al., 2005; Ducharme and Bickel, 2008). However, the presence of actin (microfilaments), vimentin (intermediate filaments), and their associated proteins (plasmin L and cofilin), instead of tubulin (microtubules), in this study could be indicative of the unexpected role of microfilaments and intermediate filaments in the growth and transport of LD in mammary epithelial cells. Actin was concentrated in the apical region of mammary epithelial cells and localized along the cytoplasmic surface of LD. Actin was suggested to help package LD with plasma membrane during budding to milk (Mather and Keenan, 1998). Beta-actin, the isoform also observed in this study, was shown to bind to intracellular LD in rat adrenocortical cells and adipocytes (Fong et al., 2001). Thus, one could assume that in the apical region of the cell, actin may bind to LD before its secretion and thereby regulate LD transport and growth, possibly by being involved in LD fusion as microtubule proteins do.

Unexpectedly, host defense proteins, mainly cathelicidins, differed between the large and small MFG fractions (Table 5). Cathelicidins have been thought to mainly originate from neutrophils, which are the major type of somatic cells in milk (Smolenski et al., 2011). Contamination of the large MFG fraction with neutrophils may therefore seem to be an obvious reason for the high level of cathelicidins. However, myeloperoxidase, the most abundant protein in neutrophils (Haegens et al., 2008), was not identified in the present study. In addition, the number of neutrophils should be low due to the 3 washing and centrifugation steps used to isolate the MFG fractions. Cathelicidin 1 ranked 15th based on the signal intensity of the 157 proteins identified in this study, which suggests that the origin of these cathelicidins might not only be neutrophils but also epithelial cells in the mammary gland. Cathelicidin-related antibacterial polypeptides have also been identified in the murine mammary gland (Murakami et al., 2005). However, to our knowledge, the expression of cathelicidins in bovine mammary gland has not been studied. Another possible reason for the selective accumulation of cathelicidins in MFGM could be due to their physical characteristics. Cathelicidins are normally positively charged and tend to fold into amphipathic structures (Zanetti, 2004), which could be the rationale for their binding to MFGM. Furthermore, this could also explain the altered affinity of cathelicidins to MFG of different sizes and therefore their higher content in the large compared with the small MFG fraction. However, this needs to be further elucidated.

Both cholesterol synthesis enzymes (LSS and NSDHL) were present in higher concentrations in the small MFG fraction (Table 6). Cholesterol is known to be an essential component for membrane integrity, permeability, rigidity, and functionality. Both LSS and NSDHL have been found to localize in intracellular LD in yeast and adipocytes (Caldas and Herman, 2003; Ohashi et al., 2003; Mullner et al., 2004; Goodman, 2009). These observations raised the possibility that LD could be the site of lipid/cholesterol synthesis rather than only being a central lipid depot (Caldas and Herman, 2003). It is possible that LD function as the site of lipid/cholesterol synthesis. If so, this could explain the higher concentration of LSS and NSDHL in the small MFG fraction because it requires relatively more membrane material, and therefore more cholesterol, to cover small MFG than large MFG. This is in agreement with the higher cholesterol content in smaller lipid droplets (Table 3). However, whether these enzymes in LD contribute to the synthesis of lipids needs to be further investigated.

In the present study, several hypotheses have been raised regarding different proteins and lipids that might be associated with MFG size. However, other reasons

may also have led to these differences. Further targeted research is required to study these possible relations, and reveal the role of the observed components in MFG formation and secretion.

Aside from gaining a better understanding of lipid synthesis and secretion, the differences in protein and lipid composition between small and large MFG may also be interesting for producing dairy products with distinctive qualities. For example, butter with low SFA and low cholesterol could be produced from small MFG and large MFG, respectively. Also, specific functional proteins could be obtained from different sized MFG. For example, immune-active proteins from milk have been suggested as potential ingredients for functional foods (Zanetti, 2004).

CONCLUSIONS

The observations from this study clearly demonstrate the difference in protein and lipid composition of the membranes of the small and large MFG fractions. Our results on PAT proteins are in agreement with their previously reported role in MFG formation and secretion. In addition, our results for the first time suggest the importance of cytoplasmic vesicle proteins, cytoskeleton proteins, and cholesterol synthesis enzymes in MFG formation and secretion. Proteomics and lipidomics analysis provides us a more complete overview of the difference in different sized MFG fractions. In future, cell biology studies focusing on the proteins and lipids discussed in this paper, including their interaction, could further elucidate the processes underlying the difference in fat globule size, and thereby lead to a better understanding of MFG formation and secretion.

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