Developmental Changes in the Milk Fat Globule Membrane Proteome During the Transition from Colostrum to Milk¹

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ABSTRACT

Shotgun proteomics, using amine-reactive isobaric tags (iTRAQ), was used to quantify protein changes in milk fat globule membranes (MFGM) that were isolated from d 1 colostrum and compared with MFGM from d 7 milk. Eight Holstein cows were randomly assigned to 2 groups of 4 cow sample pools for a simple replication of this proteomic analysis using iTRAQ. The iTRAQ labeled peptides from the experiment sample pools were fractionated by strong cation exchange chromatography followed by further fractionation on a microcapillary high performance liquid chromatograph connected to a nanospray-tandem mass spectrometer. Data analysis identified 138 bovine proteins in the MFGM with 26 proteins upregulated and 19 proteins downregulated in d 7 MFGM compared with colostrum MFGM. Mucin 1 and 15 were upregulated greater than 7-fold in MFGM from d 7 milk compared with colostrum MFGM. The tripartite complex of proteins of adipophilin, butyrophilin, and xanthine dehydrogenase were individually upregulated in d 7 MFGM 3.4-, 3.2-, and 2.6-fold, respectively, compared with colostrum MFGM. Additional proteins associated with various aspects of lipid transport synthesis and secretion such as acyl-CoA synthetase, lanosterol synthase, lysophosphatidic acid acyltransferase, and fatty acid binding protein were upregulated 2.6- to 5.1-fold in d 7 MFGM compared with colostrum MFGM. In contrast, apolipoproteins A1, C-III, E, and A-IV were downregulated 2.6- to 4.3-fold in d 7 MFGM compared with colostrum MFGM. These data demonstrate that quantitative shotgun proteomics has great potential to provide new insights into mammary development.

Key words: milk fat globule membrane, proteomics, mammary gland, mastitis

INTRODUCTION

Proteomics is a tool that will help identify proteins important to milk production and secretion. Identification of proteins associated with various aspects of milk production and secretion will provide a foundation for new research in lactation biology. Most of proteomic studies conducted thus far on mammary epithelial cells, organelles, membranes, and the secretion processes are focused on breast cancer, rodent lactation, or both (Wu et al., 2000a,b; Quaranta et al., 2001; Charlwood et al., 2002; Pucci-Minafra et al., 2002; Fortunato et al., 2003; Jacobs et al., 2004; Davies et al., 2006). Although these studies have advanced our understanding of mammary function and milk secretion, they may not address the unique aspects of milk secretion in dairy cattle. Recently, 2 papers on bovine mammary proteomics have appeared (Daniels et al., 2006; Reinhardt and Lippolis, 2006). The first paper examined mammary protein expression in growing virgin heifers (Daniels et al., 2006), and the second was a survey of proteins expressed in milk fat globule membranes (Reinhardt and Lippolis, 2006).

Our understanding of the molecular mechanisms critical to milk fat secretion is incomplete (Mather and Keenan, 1998a,b; Keenan, 2001). The scarcity of information is due in large part to the lack of cell lines that secrete milk and milk fat (Keenan, 2001). The MFGM is a rich source of membrane proteins, and proteomic analysis of these membranes has highlighted some of the possible signaling and secretory pathways used by the mammary gland (Reinhardt and Lippolis, 2006). Furthermore, the proteome of the MFGM provides additional insight into this membrane's cellular origin. The most widely accepted source of membrane for the MFGM is the apical membrane of the secretory cell (Mather and Keenan, 1998a; Keenan, 2001). Their conclusions are supported by biochemical, electron microscopy, and immunocytochemical evidence.

The major proteins in the MFGM have been identified using traditional biochemical approaches (Mather, 2000). These methods are slow, laborious, and address only one protein at a time. Proteomic and microarray approaches can identify gene and protein

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connections to a pathway that is not apparent or predictable from biochemical and genetic analysis of a biological system (Patterson and Aebersold, 2003). This approach has been applied widely to quantitative proteomics (Ross et al., 2004; DeSouza et al., 2005; Chen et al., 2006; Hu et al., 2006; Keshamouni et al., 2006; Lippolis et al., 2006) and overcomes many of the problems of 2-dimensional electrophoresis in the study of membrane proteins (Gu et al., 2003; Peirce et al., 2004; Lippolis et al., 2006).

We used a shotgun proteomics approach using amine-reactive isobaric tags (**iTRAQ**) to quantify protein changes in milk fat globule membranes (**MFGM**) that were isolated from d 1 colostrum compared with d 7 milk. Our objective was to examine how the transition from colostrum secretion to milk secretion changes protein expression in MFGM.

MATERIALS AND METHODS

Animals and Milk Fat Membrane Preparation

Colostrum or milk was collected from 8 Holstein cows at parturition and d 7 of lactation. The colostrum or milk from each collection was brought immediately to the lab and centrifuged at $10,000 \times g$ for 15 min at 4°C. The floating milk fat pellet was removed, mixed with 10 volumes of ice-cold phosphate buffered saline (pH 7) + complete protease inhibitor cocktail from Boehringer Mannheim (Indianapolis, IN), and centrifuged at $10,000 \times g$ for 15 min. This washing step was repeated 3 times until the supernatant was clear (Reinhardt and Lippolis, 2006).

The MFGM were prepared from the washed milk fat as previously described (Reinhardt et al., 2000; Prapong et al., 2005; Reinhardt and Lippolis, 2006). Washed milk fat from colostrum or milk was diluted in 10 volumes of buffer A, which contained Tris-HCL (10 mM), $MgCl_2$ (2 mM), phenylmethylsulfonyl fluoride (0.1 mM), EDTA (1 mM), 4 $\mu g/mL$ of aprotinin, and 4 $\mu g/mL$ of leupeptin at pH 7.5. The sample was homogenized using a Polytron PT-10 homogenizer (Brinkman Instruments, Boston, MA) running at 12,000 rpm. Each homogenization step was for 12 s with 30 s of sample cooling between each homogenization run. A total of three 12-s homogenizations were performed on the sample. The homogenate was mixed with an equal volume of buffer B (buffer A plus 300 mM KCl) and centrifuged at $100,000 \times g$ for 1 h. The supernatant was discarded and the membrane pellet was resuspended in buffer C (buffer A plus 150 mM KCl) (Reinhardt et al., 2000). The resuspended membrane preparation was centrifuged at $100,000 \times g$ for 1 h. The supernatant was discarded and MFGM pellet was resuspended in buffer A. Protein concentration

was determined using the BioRad Protein Assay Kit using a BSA standard. The MFGM were stored at -70°C until needed.

Extraction of Extrinsic Proteins to Concentrate MFGM Intrinsic Proteins

The MFGM prepared as described above were pelleted by centrifugation at $100,000 \times g$ for 1 h. All procedures were done at 4°C. The membrane pellet was resuspended in a small amount of 300 mM sucrose, 10 mM Tris-HCL at pH 7.5. This suspension was diluted with ice-cold 0.1 M sodium carbonate (pH 11.5) to a protein concentration 0.01 mg/mL (Fujiki et al., 1982; Reinhardt and Lippolis, 2006). The sample was incubated on ice for 1 h to extract extrinsic proteins and then centrifuged at $100,000 \times g$ for 1 h through a cushion of 300 mM sucrose (10% of the tube volume). The MFGM intrinsic protein pellet was resuspended in buffer A. Protein concentration was determined, and the extracted MFGM were stored at -70°C until needed. This procedure enriched the MFGM preparation for intrinsic proteins by reducing but not eliminating extrinsic protein content.

Sample Preparation for Mass Spectroscopy Analysis

Samples were randomized, and 4 sample pools were created (Figure 1). One hundred micrograms of protein from each sample pool was dried and processed as follows. Membrane proteins from the 4 samples were each resuspended in 50 μL of 25 mM triethyl-ammonium bicarbonate (pH 8) in 1.5-mL microcentrifuge tubes. After adding cap locks, the proteins were thermally denatured at 90°C for 20 min as described (Park and Russell, 2000, 2001). The samples were then cooled on ice for 10 min and then dried in a vacuum centrifuge. For trypsin digestion, 25 μL of proteomic-grade trypsin (20 $\mu g/mL$ in 25 mM triethyl-ammonium bicarbonate) was added to each sample. We added acetonitrile (**ACN**) so that the solution was 30% ACN (Russell et al., 2001). This ACN/trypsin solution was incubated at 37°C overnight. The next day the samples were cooled to room temperature and then dried in a vacuum centrifuge. The samples were stored dry at -20°C until used.

Each sample was then labeled using the iTRAQ kit for amine-modifying labeling reagents for multiplexed relative and absolute protein quantitation (Applied Biosystems, Foster City, CA). See Figure 1 for the sample analysis work flow. The iTRAQ-labeled peptides were dried and resuspended in 300 μL of 20 mM formic acid and 20% ACN). Samples were run on a

Figure 1. Experimental workflow. MFGM = milk fat globule membrane.

strong cation exchange (SCX) column (Mono S PC 1.6/5; Amersham, Piscataway, NJ) with a gradient of solution A (20 mM formic acid; 20% ACN, pH 2.7) and solution B (20 mM formic acid; 20% ACN; 350 mM ammonium bicarbonate, pH 4.7). Sample fractions (0.5 mL) were collected over a 15-min gradient of 0 to 35% solution B followed by 1.5 min in 90% solution B. Sample fractions were dried down and resuspended in 30-

mL 0.1% formic acid in 5% ACN (Lippolis and Reinhardt, 2005; Lippolis et al., 2006).

HPLC and Tandem Mass Spectroscopy of the Samples

Each SCX fraction was analyzed by capillary HPLC (CapLC; Waters, Milford, MA) in line with a Q-TOF

Ultima API mass spectrometer (Waters). An Altantis C₁₈ NanoEase column (75 mm × 100 mm) was used for peptide separation. The system was configured to concentrate and wash the injected sample on a Symmetry 300 C₁₈ precolumn. Seven minutes after the start of sample loading, the precolumn was switched in line with the analytical column to allow the trapped peptides to be eluted onto the analytical column. Mobile phase A was 0.1% formic acid in 5% ACN. Mobile phase B was 0.1% formic acid in 95% ACN. The gradient was 95% A for 5 min and then ramped linearly to 60% A over 85 min. Over the next 2.5 min it was ramped to 10% A and held an additional 10 min before reequilibration of the column. The flow rate was approximately 300 nL/min. The analytical column was connected to Waters lockspray-nanospray interface on the front of the mass spectrometer. The lockspray used the peptides [Glu¹]-fibrinopeptide B and leucine enkephalin (Sigma, St. Louis, MO) as mass calibration standards. The capillary voltage was 3,500 V and was tuned for signal intensity. The 5 most intense ions with charge states between 2 and 4 were selected in each survey scan if they met the switching criteria. Three collision energies were used to fragment each peptide ion based on its mass to charge (*m/z*) values. Each fraction was run 4 times, once collecting MSMS data on the full range of parent masses followed by runs collecting MSMS data on parent masses in 3 mass ranges (400 to 635, 635 to 750, and 750 to 1,500; Lippolis et al., 2006) for a total 112 runs.

Protein Identification

All MS data files were processed into *pkl* files using ProteinLynx Global Server 2.0 (Waters, Milford, MA) and lock-spray correction of MS data with [Glu]-fibrinopeptide B and MSMS with leucine enkephalin. The *pkl* files were merged into a single file using the program Merge (Matrix Science, London, UK). The data were then analyzed with Mascot (Matrix Science) using the NCBI NR protein database with mammalia taxonomy, MMTS (C), iTRAQ (K), and iTRAQ (N-term) as fixed modifications, and iTRAQ (Y) and oxidation (M) as variable modifications. The peptide tolerance was 20 ppm and the MSMS tolerance was 0.05 Da. The Mowse scoring algorithm (Pappin et al., 1993) in Mascot was used to determine the probability that a protein was correctly identified. We used $P < 0.05$ for protein identity and a Mowse score greater than 41 yields a $P < 0.05$ protein identity. Abundance data were obtained by determination of the peak area of each of the iTRAQ labels after the peak was smoothed and centroided. Data were extracted from the Mascot.-dat file with a Perl script that parsed peptide data

including the abundance numbers for iTRAQ daughter ion peaks within a ± 0.05 Da tolerance. Data were manually organized to remove duplication of query assignments and duplicate proteins. All proteins without at least two MSMS spectra with a probability of a correct match greater than 95% were removed. Only MSMS spectra with abundance information for all 4 iTRAQ labels were retained. Relative changes in protein expression were calculated in the same way as described by Ross et al. (2004). Briefly, the abundance of the iTRAQ label used to tag either the colostrum MFGM sample pool 1 was divided by the sum of the abundance of the colostrum MFGM sample pool 1 + d 7 MFGM sample pool 1. The range of this calculation is between 0 and 1 with result of 0.5 meaning no change in protein expression. So a low Ross ratio indicated up-regulation and a high Ross ratio indicated down-regulation in d 7 MFGM compared with colostrum MFGM. The procedure was used for the simple replicate data for sample pool 2. The mean Ross calculation for all MSMS spectra for these simple replicate samples was 0.52 with a standard deviation of 0.14. A change was considered real if the average iTRAQ calculation of a protein was outside the range of the total MSMS Ross ratio plus or minus the standard deviation (Ross et al., 2004), which for these data equates to Ross ratios of 0.38 to 0.66.

Gel Electrophoresis and Western Blotting

The MFGM proteins from individual cows were incubated for 5 min at 95°C in a modified Laemmli buffer containing 150 mg/mL urea and 65 mM DTT. Equal volumes of sample were loaded into 2 wells of an 8 to 16% Tris-glycine gradient gels (Novex, San Diego, CA) and electrophoresed for 1.5 h at 125 V. Proteins were transferred to nitrocellulose membranes for 75 min at 25 V in 0.192 M glycine, 0.025 M Tris at pH 8.3 (Reinhardt et al., 2004a,b). All antibodies were diluted according to manufacturer's instructions. Antibodies to xanthine oxidase (ab6194), syntaxin 3 (ab4113), and actin (ab8226) were purchased from Abcam (Cambridge, MA). SNAP23 (Pai-738) antibody was purchased from Affinity BioReagents (Golden, CO). The bovine TLR-2 antibody was prepared from the C-terminus peptide using procedures described (Reinhardt et al., 2000). Blots were developed using Pierce's Supersignal (Pierce Products, Rockford, IL) according to the protocol provided by the manufacturer.

RESULTS

Figure 2 showed a representative 1-DE gel image for the carbonate extracted colostrum and d 7 MFGM

Figure 2. Carbonate-extracted colostrum milk fat globule membranes (MFGM; C) and 7-d milk MFGM separated on an 8 to 16% SDS-PAGE gel from 3 of the 8 cows used in this study. Each lane represents 25 μ g of membrane protein with the location of molecular weight markers indicated at the left side of the gel.

for 3 cows. Gross differences in the proteins expressed can be seen between colostrum and d 7 milk MFGM. Using a shotgun proteomics approach outlined in Figure 1, we identified and have expression data for greater than 130 proteins. The false discovery rate for our data was 1.29% as determined by using a decoy database, thus yielding high confidence in our identification data. Approximately 70% of the proteins identified are membrane-associated proteins with the remainder being secreted proteins or of cytosol origin. Data analysis identified 26 proteins upregulated and 19 proteins downregulated in d 7 MFGM compared with colostrum MFGM (Tables 1 and 3, respectively). A change was considered real if the average iTRAQ calculation of a protein was outside the range of the average of all MSMS Ross ratios plus or minus the standard deviation (Ross et al., 2004), which for these data equates to Ross ratios of 0.38 to 0.66 (see Materials and Methods section). Table 2 shows proteins considered unchanged using the criteria of Ross. However, the reader will note that the data in Table 2 showing fold changes up, down, or no change are noted based on mean Ross ratios. These data are presented for information only as some of the changes may prove to be real as described for syntaxin 3 and TLR2 below.

Proteins associated with various aspects of lipid transport synthesis and secretion such as acyl-CoA synthetase, lanosterol synthase, lysophosphatidic acid acyltransferase, cell death-inducing DFFA-like effector A, and fatty acid binding protein were upregu-

lated 2.6- to 5.1-fold in d 7 MFGM compared with colostrum MFGM (Table 1). The mucin proteins, mucin 1 and 15, showed the greatest expression change of all the proteins analyzed. Both mucin 1 and 15 were upregulated greater than 7-fold in MFGM from d 7 milk compared with colostrum MFGM (Table 1).

Specific proteins involved with vesicle transport and protein trafficking showed variable regulation. Proteins, such as SAR1a gene homolog 1, SNAP-23, synaptosomal-associated protein 29 were upregulated 2- to 3-fold in MFGM from d 7 milk compared with colostrum MFGM (Table 1). However, many proteins involved in vesicle transport and protein trafficking have borderline expression changes using the criteria of Ross et al., 2004 (Table 2). To examine these proteins further, we performed Western blots (Figure 3) on individual cow samples using antibodies to xanthine dehydrogenase SNAP-23, syntaxin 3, and TLR2, which were shown to be upregulated 2.6-, 3.0-, 1.6-, and 1.7-fold, respectively, in MFGM from d 7 milk compared with colostrum MFGM (Tables 1 and 2). Figure 3 (lower panel) shows the comparison results for Western blot data from 8 individuals versus iTRAQ expression results for two 4-cow sample pools. The result compare quite favorably. Syntaxin 3 and TLR2, which appeared to be upregulated in MFGM from d 7 milk compared with colostrum MFGM (Table 2) but did not meet the criteria of Ross et al. (2004) for a change in expression, can be seen in Figure 3. By Western blotting syntaxin 3 and TLR2 were upregulated in MFGM from d 7 milk compared with colostrum MFGM. The Western blot data confirm the validity of the iTRAQ data as well as demonstrating that the Ross criteria for protein expression changes is conservative. This gives the changes noted in Tables 1 and 3 more confidence despite the limitations of proteomic iTRAQ experiments with regard to experiment replicates and suggests that proteins in Table 2 that are up- or down-regulated 1.6-fold or greater may be real changes as was found for syntaxin 3 and TLR2 by Western blotting.

As shown in Table 3 secreted proteins such as apolipoproteins A1, C-III, E, A-IV clusterin and lactoferrin were downregulated 2.6- to 4.3-fold in d 7 MFGM compared with colostrum MFGM (Table 3).

DISCUSSION

Shotgun proteomics in conjunction with iTRAQ protein expression tags were used to identify and measure developmental protein expression changes in the MFGM isolated from colostrum vs. MFGM isolated from milk on d 7 of lactation. The goal of this experimental approach is to ultimately make connections

Table 1. Proteins upregulated in milk fat globule membranes (MFGM) on d 7 compared with colostrum MFGM according to the method of Ross et al. (2004)

NCBI accession #	Mowse score	Protein name	Fold change on d 7	Ross mean pool 1	SD pool 1	Ross mean pool 2	SD pool 2	MS/MS # ¹
gi 41386778	114	Mucin 1	7.7↑	0.15	0.04	0.12	0.05	22
gi 41386723	192	Mucin 15	7.4↑	0.17	0.11	0.23	0.14	8
gi 27805809	450	Fatty acid binding protein	5.1↑	0.17	0.09	0.25	0.11	27
gi 741536	369	Lactophorin	4.7↑	0.22	0.07	0.19	0.08	26
gi 76651893	237	Cell death-inducing DFFA-like effector a	4.4↑	0.26	0.10	0.20	0.10	18
gi 76607229	271	ATPase type 13A4	4.4↑	0.21	0.09	0.22	0.08	7
gi 76639775	246	Solute carrier family 7 (cationic amino acid transporter, 7+ system)	4.0↑	0.24	0.10	0.25	0.10	23
gi 27806507	988	5'-nucleotidase, ecto	3.8↑	0.25	0.09	0.23	0.10	67
gi 27806759	2634	Adipose differentiation-related protein	3.4↑	0.28	0.10	0.27	0.10	377
gi 76655934	271	Lysophosphatidic acid acyltransferase	3.3↑	0.26	0.11	0.25	0.07	6
gi 27806233	3050	Butyrophilin	3.2↑	0.26	0.10	0.28	0.09	601
gi 76656379	176	SAR 1 a gene homolog 1	3.0↑	0.27	0.11	0.28	0.04	9
gi 76683695	561	ATP-binding cassette, subfamily G, member 2	3.0↑	0.28	0.09	0.26	0.08	68
gi 76678893	90	SNAP-23	3.0↑	0.28	0.14	0.36	0.10	4
gi 76607778	321	Lanosterol synthase	2.8↑	0.26	0.06	0.30	0.06	13
gi 76624916	123	Ciliary neurotrophic factor receptor alpha	2.7↑	0.35	0.16	0.31	0.10	5
gi 76681278	1179	Pancreatic secretory granule membrane major glycoprotein GP2	2.7↑	0.4	0.06	0.24	0.10	141
gi 27806775	4464	Xanthine dehydrogenase	2.6↑	0.28	0.09	0.32	0.09	594
gi 27807195	888	Member 2	2.6↑	0.27	0.08	0.33	0.07	75
gi 76634086	233	Acyl-CoA synthetase long-chain family member 3	2.6↑	0.32	0.13	0.36	0.11	9
gi 76611288	137	Proto-oncogene tyrosine-protein kinase	2.6↑	0.31	0.13	0.35	0.17	5
gi 76620196	333	Protein kinase, cGMP-dependent, type II	2.6↑	0.36	0.10	0.37	0.12	14
gi 76674545	336	NAD(P) dependent steroid dehydrogenase	2.5↑	0.28	0.14	0.37	0.12	16
gi 76639754	138	Synaptosomal-associated protein 29	2.2↑	0.42	0.01	0.38	0.12	3
gi 76655661	192	Acyl-CoA synthetase long-chain family member 1	2.1↑	0.37	0.10	0.37	0.16	10
gi 76626756	273	Dehydrogenase/reductase	2.0↑	0.33	0.10	0.38	0.08	14

¹MS/MS # is the number of peptides from this protein that were sequenced in the mass spectrometer and used for identification. For abundant proteins such as "butyrophilin" this number is very large due to repeated sequencing of the same peptides.

between cellular functions and pathways that may not be predictable from traditional biochemical experiments in lactation biology.

The extreme dynamic range seen in protein abundances in general complicates proteomic analysis. It is estimated that cells may contain proteins with as few as 10 copies per cell ranging up to ~1,000,000 copies per cell (Moritz et al., 2004). For this reason it is important to simplify the proteome before analysis. We did this, in part, by extracting the MFGM with carbonate to remove extrinsic proteins (Fujiki et al., 1982), so we could then focus on a simpler MFGM proteome that was enriched for intrinsic proteins. However, for the bovine MFGM proteome, one of the greatest obstacles to the identification of lower abundance proteins is the abundance of a few MFGM proteins, such as butyrophilin (Mather, 2000). Butyrophilin constitutes 30 to 40% of the total protein in the Holstein MFGM (Mather, 2000). Due to this dominate protein and its protein partners, adipophilin and xanthine oxidase, excessive peptide signal results in the mass spectrometer missing many coeluting low abundance proteins. Several of these abundant MFGM proteins contribute to this problem in the analysis of the MFGM proteome as they account for >70% of the high quality peptides seen by the mass spectrometer in the

shotgun MFGM proteome. The consequence of these or any overly abundant proteins in a proteome is simply a reduction in the total number of proteins that can be identified with high confidence. Future advancements into the analysis of less abundant proteins of the MFGM proteome will require specific depletion of high abundant proteins as has been used successfully in blood serum proteomic studies.

A detailed discussion of all the protein changes is beyond the scope of this paper. We will discuss some changes related to fat metabolism, the secretory process and factors important for mammary health, but provide the bulk of the data for building a developmental MFGM proteome database.

The tripartite complex of proteins of adipophilin, butyrophilin, and xanthine dehydrogenase (Heid et al., 1996; McManaman et al., 2002; Vorbach et al., 2002; Ogg et al., 2004) were individually upregulated in d 7 MFGM in parallel compared with colostrum MFGM (Table 1). This parallel upregulation is expected for a complex with 3 components fixed in a molar ratio. These proteins and other proteins associated with lipid transport, synthesis, and secretion are upregulated several-fold in d 7 MFGM compared with colostrum MFGM (Table 1), which is indicative of an

Table 2. Proteins unchanged in milk fat globule membranes (MFGM) on d 7 compared with colostrum MFGM according to the method of Ross et al. (2004)¹

NCBI accession #	Score	Protein name	Fold change on d 7	Ross mean pool 1	SD pool 1	Ross mean pool 2	SD pool 2	MS/MS #
gi 76671278	306	Vesicle amine transport protein 1	1.8↑	0.37	0.06	0.35	0.05	10
gi 76621988	613	Syntaxin binding protein 2	1.8↑	0.36	0.11	0.39	0.09	23
gi 76635818	848	Milk folate binding protein	1.8↑	0.35	0.07	0.39	0.08	107
gi 32189338	1,135	Polymeric immunoglobulin receptor	1.8↑	0.42	0.07	0.37	0.12	128
gi 76624275	570	Rab-18	1.8↑	0.35	0.09	0.42	0.07	48
gi 76646062	316	Guanine nucleotide binding protein (G protein), alpha 13	1.7↑	0.38	0.04	0.37	0.04	9
gi 76615473	165	SNARE protein Ykt6	1.7↑	0.35	0.10	0.45	0.09	6
gi 76653198	96	3 beta-hydroxy-delta 5-C27-steroid oxidoreductase	1.7↑	0.34	0.06	0.42	0.04	3
gi 27806379	678	Toll-like receptor 2	1.7↑	0.36	0.06	0.40	0.06	26
gi 76686605	108	RAB2	1.7↑	0.33	0.06	0.48	0.13	6
gi 76669446	116	Phospholipid scramblase 1	1.6↑	0.41	0.06	0.37	0.10	3
gi 76636653	208	Syntaxin 3	1.6↑	0.38	0.12	0.46	0.19	7
gi 76669092	94	Signal peptide peptidase-like 2A	1.6↑	0.44	0.06	0.36	0.06	6
gi 76626930	206	Peptidylprolyl isomerase A	1.5↑	0.4	0.07	0.43	0.10	12
gi 68299807	410	Eukaryotic translation elongation factor 1 alpha 1	1.5↑	0.43	0.09	0.42	0.08	21
gi 76629653	247	RAB1	1.4↑	0.39	0.12	0.52	0.09	16
gi 76659124	379	Calpain 6	1.4↑	0.36	0.07	0.50	0.06	11
gi 76639295	135	RAB35	1.4↑	0.39	0.02	0.45	0.03	5
gi 76621819	186	RAB3	1.4↑	0.41	0.09	0.46	0.09	8
gi 27805889	375	Guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O	1.3↑	0.38	0.06	0.51	0.05	12
gi 76658481	195	Rab-5A	1.3↑	0.43	0.08	0.48	0.06	10
gi 30794318	445	Isocitrate dehydrogenase 1	1.3↑	0.45	0.10	0.51	0.12	15
gi 76669596	94	Ral-A	1.3↑	0.4	0.07	0.51	0.07	11
gi 27805979	191	Lactalbumin, alpha	1.3↑	0.43	0.07	0.49	0.06	9
gi 28603778	194	ADP-ribosylation factor 1	1.2↑	0.44	0.07	0.52	0.10	26
gi 76638403	86	ELMO domain containing 2	1.2↑	0.52	0.12	0.49	0.11	5
gi 76681914	499	Ig alpha-2 chain C region	1.2↑	0.49	0.05	0.44	0.08	27
gi 76660725	122	Rab-21	1.2↑	0.39	0.06	0.55	0.06	4
gi 27807095	433	Solute carrier family 5 (sodium/glucose cotransporter), member 1	1.2↑	0.45	0.08	0.55	0.13	23
gi 27807509	140	Guanine nucleotide binding protein (G protein), gamma 12	1.2↑	0.41	0.07	0.56	0.09	6
gi 76627752	90	Rab-8B	1.2↑	0.48	0.13	0.49	0.10	4
gi 76636007	159	RhoG	1.2↑	0.42	0.07	0.55	0.05	6
gi 76649136	304	Guanine nucleotide-binding protein G(i), alpha-2 subunit	1.1↑	0.44	0.07	0.52	0.07	13
gi 76636197	252	CD59 antigen p 18-20	1.1↑	0.43	0.06	0.53	0.04	64
gi 76649604	226	Rab-7	1.1↑	0.44	0.14	0.58	0.08	5
gi 42543638	211	Rac1	1.0	0.46	0.06	0.56	0.13	14
gi 76681832	483	Ig kappa chain C region	1.0	0.59	0.05	0.48	0.11	35
gi 27807289	208	Annexin A2	1.0	0.55	0.10	0.57	0.19	10
gi 76637055	322	Guanine nucleotide-binding protein G(q), alpha subunit	1.0	0.51	0.14	0.56	0.10	12
gi 41386760	307	CD14	1.0	0.49	0.08	0.51	0.07	10
gi 76611597	261	Cell division cycle 42	1.0	0.44	0.10	0.61	0.07	12
gi 76661612	507	Concentrative Na+-nucleoside cotransporter	1.0	0.47	0.07	0.56	0.07	56
gi 76668089	102	Myristoylated alanine-rich C-kinase substrate	1.0	0.45	0.05	0.57	0.03	4
gi 27806443	229	Rac1	1.0	0.47	0.06	0.58	0.11	14
gi 60592790	715	Fatty acid synthase	1.0	0.53	0.08	0.51	0.09	19
gi 27806081	109	Peroxiredoxin 1	1.1↓	0.52	0.19	0.54	0.21	7
gi 66792754	136	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	1.1↓	0.54	0.06	0.50	0.04	6
gi 76621936	214	RAB11	1.1↓	0.53	0.15	0.59	0.14	5
gi 76653953	235	Guanine nucleotide-binding protein, beta 2	1.1↓	0.49	0.08	0.58	0.07	16
gi 76616512	140	Glyceraldehyde-3-phosphate dehydrogenase	1.1↓	0.47	0.06	0.59	0.00	3
gi 76621720	302	Basigin	1.1↓	0.54	0.07	0.53	0.06	6
gi 27805925	811	Heat shock 70 kDa protein 8	1.1↓	0.51	0.08	0.59	0.08	34
gi 27806713	802	CD36	1.1↓	0.49	0.06	0.62	0.07	147
gi 27806765	606	Actin, beta	1.2↓	0.53	0.09	0.61	0.13	19
gi 76617076	630	NADH-cytochrome b5 reductase	1.2↓	0.51	0.07	0.61	0.10	26
gi 76657186	152	Serum amyloid A1	1.2↓	0.54	0.07	0.60	0.14	25
gi 76658444	302	CD81	1.2↓	0.48	0.05	0.66	0.06	27
gi 41386719	2,766	Lactadherin	1.2↓	0.53	0.07	0.61	0.07	838
gi 76609703	126	Megalin	1.3↓	0.64	0.22	0.58	0.14	5
gi 27807503	210	Ribosomal protein S27a	1.3↓	0.52	0.11	0.66	0.11	10
gi 76652523	155	Hemoglobin alpha chain	1.3↓	0.5	0.08	0.69	0.03	6
gi 76640813	693	Haptoglobin	1.4↓	0.6	0.08	0.58	0.08	51

Continued

Table 2 (Continued). Proteins unchanged in milk fat globule membranes (MFGM) on d 7 compared with colostrum MFGM according to the method of Ross et al. (2004)¹

NCBI accession #	Score	Protein name	Fold change on d 7	Ross mean pool 1	SD pool 1	Ross mean pool 2	SD pool 2	MS/MS #
gi 60592792	105	Heat shock 90kD protein 1, alpha	1.4↓	0.57	0.13	0.64	0.13	5
gi 28461271	304	RAP1	1.4↓	0.53	0.08	0.65	0.06	19
gi 76654370	85	Tweety 3	1.4↓	0.55	0.08	0.64	0.15	5
gi 76658766	76	Chloride intracellular channel protein 4	1.4↓	0.53	0.02	0.65	0.09	3
gi 76670195	405	Monocarboxylate transporter 1	1.4↓	0.59	0.04	0.58	0.05	8
gi 28603756	347	Rho-a	1.4↓	0.54	0.06	0.66	0.06	19
gi 76690221	187	Calnexin	1.4↓	0.58	0.07	0.62	0.06	6
gi 61820437	75	Saccharopine dehydrogenase	1.5↓	0.6	0.07	0.61	0.10	6
gi 61873314	174	Tubulin alpha-2	1.5↓	0.61	0.03	0.60	0.04	4
gi 76632584	140	Rab GDP dissociation inhibitor beta	1.6↓	0.53	0.05	0.72	0.06	6
gi 71037405	90	Heat shock 27kDa protein 1	1.6↓	0.6	0.05	0.63	0.05	6
gi 28461193	761	Annexin I	1.6↓	0.65	0.06	0.71	0.10	40
gi 27806351	327	Villin 2	1.6↓	0.56	0.09	0.70	0.08	12
gi 27807091	203	Solute carrier family 2 (facilitated glucose transporter), member 3	1.6↓	0.64	0.12	0.69	0.11	6
gi 27807083	110	Solute carrier family 1, member 1	1.6↓	0.52	0.01	0.77	0.07	3
gi 42476303	207	Toll-like receptor 4	1.6↓	0.57	0.06	0.69	0.08	8
gi 27806963	352	Casein alpha-S2	1.7↓	0.65	0.05	0.63	0.05	20
gi 27806911	244	CD9	1.7↓	0.59	0.09	0.72	0.11	22
gi 76673946	138	G protein-coupled receptor family C, group 5, member C isoform a	1.7↓	0.57	0.05	0.73	0.06	4
gi 76674964	309	Folate hydrolase 1	1.8↓	0.64	0.13	0.70	0.15	15
gi 1103842	161	Stomatin	1.8↓	0.63	0.1	0.69	0.1	9
gi 76622034	85	Complement C3	1.8↓	0.57	0.10	0.62	0.10	8
gi 27806617	83	GDP dissociation inhibitor 1	1.8↓	0.59	0.13	0.76	0.11	4
gi 76636328	110	CD82	1.8↓	0.61	0.02	0.69	0.04	3
gi 306767	193	Gamma-glutamyl transpeptidase	1.9↓	0.61	0.07	0.72	0.04	7
gi 76652249	95	Vacuolar protein sorting factor	1.9↓	0.63	0.10	0.72	0.13	6
gi 76648175	384	Programmed cell death 6 interacting protein	1.9↓	0.63	0.11	0.79	0.10	13
gi 76634215	106	Vacuolar protein sorting 28	1.9↓	0.54	0.04	0.85	0.07	4
gi 41386683	233	Beta-2-microglobulin	1.9↓	0.61	0.09	0.74	0.08	23
gi 76657058	263	Prominin 1	2.1↓	0.57	0.07	0.66	0.10	6
gi 76619354	316	Annexin A5	2.2↓	0.66	0.06	0.72	0.07	10

¹Changes are still noted, and some may be real as discussed.**Table 3.** Proteins downregulated in milk fat globule membranes (MFGM) on day compared with colostrum MFGM according to the method of Ross et al. (2004)

NCBI accession #	Score	Protein name	Fold change on d 7	Ross mean pool 1	SD pool 1	Ross mean pool 2	SD pool 2	MS/MS #
gi 76637462	141	6-Phosphogluconate dehydrogenase	2.4↓	0.74	0.17	0.82	0.13	6
gi 76676136	147	Sodium- and chloride-dependent neutral and basic amino acid transporter B(0+)	2.5↓	0.66	0.10	0.82	0.12	7
gi 76693216	320	Ig mu chain C region	2.5↓	0.75	0.05	0.69	0.06	47
gi 27806311	431	Apolipoprotein A1	2.6↓	0.71	0.09	0.76	0.08	13
gi 47564119	264	Apolipoprotein C-III	2.6↓	0.72	0.08	0.74	0.05	26
gi 27881412	295	Casein kappa	2.6↓	0.71	0.11	0.78	0.11	33
gi 76653237	89	Prostasin	2.7↓	0.71	0.06	0.77	0.09	8
gi 76622455	109	Acyl-CoA synthetase long-chain family member 6	2.8↓	0.75	0.06	0.73	0.10	6
gi 30794292	1,378	Lactoferrin	2.8↓	0.69	0.08	0.81	0.08	67
gi 27806907	688	Clusterin	2.8↓	0.75	0.12	0.78	0.13	36
gi 76641204	133	Serine protease hepsin	2.8↓	0.7	0.06	0.79	0.02	3
gi 30794348	374	Casein alpha-S1	2.9↓	0.81	0.08	0.70	0.07	36
gi 28849953	132	Seminal plasma 30K protein	3.1↓	0.74	0.02	0.78	0.05	3
gi 27806861	376	Lactoglobulin, beta	3.5↓	0.75	0.07	0.82	0.04	7
gi 27806739	466	Apolipoprotein E	3.6↓	0.76	0.07	0.81	0.06	17
gi 76635249	306	Apolipoprotein A-IV	4.3↓	0.81	0.07	0.83	0.09	7
gi 27806977	114	Fc fragment of IgG, low affinity IIa, receptor for (CD32)	4.5↓	0.79	0.06	0.85	0.07	4
gi 76669880	172	Hypothetical protein XP_613279	5.4↓	0.86	0.02	0.83	0.02	3
gi 76678688	687	Ig gamma-1 chain C region, membrane-bound form	5.6↓	0.83	0.09	0.89	0.08	71

Figure 3. Upper panel: representative Western blots of carbonate-extracted colostrum milk fat globule membranes (MFGM; C) and 7-d milk MFGM (M) separated on an 8 to 16% SDS-PAGE gel from 4 of the 8 cows used in this experiment. Cows 1 and 4 are from sample pool 1. Cows 5 and 8 are from sample pool 2. Lower panel: graphical comparison of amine-reactive isobaric tag (iTRAQ) expression data (black bars) compared with Western blot data (corrected for actin expression) from all 8 cows (gray bars). XO = xanthine dehydrogenase/oxidase.

early developmental shift in milk fat transport despite higher fat content in colostrum (Parrish et al., 1950).

The marked upregulation of cell death-inducing DFFA-like effector a (**CIDEA**) was at first puzzling as it originally identified function was apoptosis (Reed et al., 2003). Subsequent research has shown that CIDEA plays a prominent role in fat energy balance and fatty acid regulation (Zhou et al., 2003; Nordstrom et al., 2005). This protein may therefore be a new example of “gene sharing” as described for xanthine oxidase, where it is both an enzyme important in purine metabolism and separately plays a key role as a membrane-associated protein in association with adipophilin and butyrophilin with a key role in milk fat secretion (Heid et al., 1996; McManaman et al., 2002; Vorbach et al., 2002; Ogg et al., 2004). The identification of CIDEA in the MFGM and its upregulation in d 7 MFGM is an example of the power of proteomics in identifying potential new target proteins for hypothesis-driven research in lactation biology.

We found that some proteins of lipid transport and secretion, such as the lipoproteins, were downregulated in d 7 MFGM compared with colostrum MFGM. These proteins have all been previously associated with the MFGM proteome (Cavaletto et al., 2004), but the reasons for their downregulation at d 7 of lactation are currently unknown.

Mather and Keenan (1998b) point out in their review that the general progress in understanding the nature and regulation of milk secretion at the cellular and molecular level has lagged behind the progress in other secretory systems. Furthermore, much of what is proposed is by analogy with other quite different secretory model systems. Using quantitative shotgun proteomics we demonstrated that membrane/protein trafficking proteins were upregulated in MFGM by d 7 or tended to be upregulated.

The Rab proteins are low molecular weight GTP-binding proteins that form the largest branch of the Ras superfamily of GTPases. The Rab proteins and their effectors coordinate various stages of transport in the secretory pathway (Zerial and McBride, 2001). Rab18, which is up 1.8-fold in d 7 MFGM, regulates lipid droplet-associated membrane formation and interacts with adipophilin (upregulated 3.4-fold), which is involved in lipid droplet formation and secretion (Martin et al., 2005; Ozeki et al., 2005; McManaman et al., 2007). Three membrane proteins, SNAP-25, synaptobrevin, and syntaxin, form the core of a ubiquitous membrane fusion machine that interacts with the soluble proteins *N*-ethylmaleimide-sensitive factor (NSF) and α -SNAP. The Rab proteins, in coordination with the core fusion machinery and Munc-18, help to mediate vesicle docking and fusion. The SNAP are

cytosolic proteins that play a key role in the process of membrane fusion in intracellular vesicle trafficking. In eukaryotic cells, the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex is critical to membrane docking and fusion and is believed to impart some degree of specificity between vesicle SNARE and target organelle SNARE. In neurons and neuroendocrine cells, the SNARE complex consists of the integral membrane proteins VAMP (vesicle-associated membrane protein), syntaxin, and SNAP25. In nonneuronal tissues, such as mammary tissue, SNAP23 functionally replaces SNAP25 in the SNARE complex. Studies show that VAMP, syntaxin, and SNAP23 are required for SNARE function. This complex exists as a heterotrimer of the three proteins (Zerial and McBride, 2001; Bonifacino and Glick, 2004). The high expression of SNAP23 on d 7 of lactation was confirmed by Western blotting (Figure 3). Thus, the firm identification and quantitative changes in SNAP23, syntaxin 3, along with several of the Rab in purified MFGM, provide a foundation of proteins likely key for the study and understanding of secretory processes in the cow.

A number of proteins were differentially expressed in the MFGM of d 7 MFGM compared with colostrum MFGM whose functions have been associated with protection of the cow or calf from infections (Nonnecke and Smith, 1984; Hamosh et al., 1999; Mather et al., 2001; Patton, 2001; Paape et al., 2002; Cavaletto et al., 2004; Goldammer et al., 2004; Strandberg et al., 2005; Nemchinov et al., 2006; Reinhardt and Lippolis, 2006). In this class of proteins, mucin 15 and mucin 1 were the only antiinfection proteins upregulated in d 7 MFGM compared with colostrum MFGM. They were also the 2 proteins with the greatest change at greater than 7-fold up in d 7 milk (Table 1). The TLR 2, CD59, CD14, lactadherin, and TLR 4 were unchanged using the criteria of (Ross et al., 2004; Table 2). Western blot data (Figure 3) for TLR2 clearly show that this protein is upregulated ~1.7-fold in the MFGM from d 7 of lactation, which matches the results found with the iTRAQ method. This and other data discussed indicated that the Ross et al. (2004) cutoffs for protein changes are on the conservative side. There was some indication that TLR 4 is downregulated, but that conclusion will have to wait for specific antibodies for this protein. Clusterin and lactoferrin, which are known to decline in lactation (Nonnecke and Smith, 1984), were downregulated 2.8-fold in d 7 MFGM compared with colostrum MFGM.

CONCLUSIONS

These data demonstrate that quantitative shotgun proteomics has great potential to provide new insights

into mammary development. It is yielding new information about MFGM proteome and more importantly the apical membrane of the secretory cell with the ultimate goal of new hypothesis-driven research into lactation biology. Development of methods to deplete major MFGM proteins will greatly increase the numbers of proteins identified in the MFGM.

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