4

Intracellular Origin of Milk Fat Globules and the Nature of the Milk Fat Globule Membrane

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4.1. Introduction

In this chapter, we review what is known about the intracellular origin, growth, cytoplasmic transit and secretion of the lipid globules of milk, and the nature and intracellular origin of the milk fat globule membrane (MFGM). Material found in the MFGM appears to originate from the endoplasmic reticulum during the initial formation of the lipid droplet precursors of milk fat globules, and from post-Golgi membranes, including the apical plasma membrane, during the secretion of lipid droplets from the cell. Milk fat globules constitute 95%, by weight, of the lipids in cow's milk, of which more than 98% comprise triacylglycerols. The remainder of the mass of fat globules is composed of diacylglycerols, sterols, sterol esters, phospholipids, glycosphingolipids, and proteins associated with the droplet surface.

In writing this chapter we have emphasized findings since the previous edition of this book was published in 1994. Much of the new information gained in this area concerns the proteins of the MFGM. Since the previous edition, there have been few additional studies on intracellular aspects of milk lipid globule formation and on the composition of the MFGM. For those interested in earlier literature and coverage of historical aspects of research on milk lipid globules, the comprehensive review by Brunner (1974) and later reviews (Anderson and Cawston, 1975; Patton and Keenan, 1975;

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McPherson and Kitchen, 1983; Keenan *et al.*, 1988; Mather, 1987; Keenan and Dylewski, 1995; Keenan and Patton, 1995; Mather and Keenan, 1983, 1998) are recommended.

Much of the information we have presented on MFGM has been learned through studies of cows' milk. Milk fat globules and MFGM from other species, notably from humans, have been subjects of increasing study over the past 20 years. Herein, we discuss information gained from other species only where equivalent information for the cow is not available, or where information from other studies is contradictory to, or confirmatory of, what is believed about cows' milk. Extensive information about human milk fat globules can be found in books edited by Jensen (1995) and Newburg (2001).

4.2. Intracellular Origin and Growth of Milk Fat Globules

The membrane surrounding milk lipid globules essentially is a tripartite structure that originates from the apical plasma membrane, from the endoplasmic reticulum and possibly from other intracellular compartments. That portion of the MFGM originating from apical membranes, termed the primary membrane, has a typical bilayer appearance and electron-dense coat material on the inner face (Figure 4.1). The material derived from the endoplasmic reticulum has the appearance of a monolayer of proteins and polar lipids that covers the triacylglycerol-rich core lipids of the globule before secretion (Figure 4.2). This coat material compartmentalizes the core lipids within the cell and may participate in the intracellular fusion of droplets with each other. Constituents of this coat also may be involved in the interaction of droplets with the plasma membrane during the process of secretion.

Small lipid droplets that are the intracellular precursors of milk lipid globules appear to accumulate at focal points on or in the endoplasmic reticulum membrane (Dylewski *et al.*, 1984a; Zaczek and Keenan, 1990). Several studies suggest that the lipids, presumed to be primarily triacylglycerols, accumulate between the outer and inner halves of the bilayer and are then released into the cytosol as droplets coated with the cytoplasmic half of the endoplasmic reticulum membrane (Patton and Keenan, 1975; Zaczek and Keenan, 1990; Keenan *et al.*, 1992; Mather and Keenan, 1998). Storage lipid droplets in adipocytes and other cells also appear to be released from the endoplasmic reticulum into the cytosol in like manner (Murphy and Vance, 1999; Murphy, 2001). Using a cell-free system, Keenan *et al.* (1992) showed that lipid droplets are released from mammary gland endoplasmic reticulum bound to nitrocellulose membrane and that these droplets in

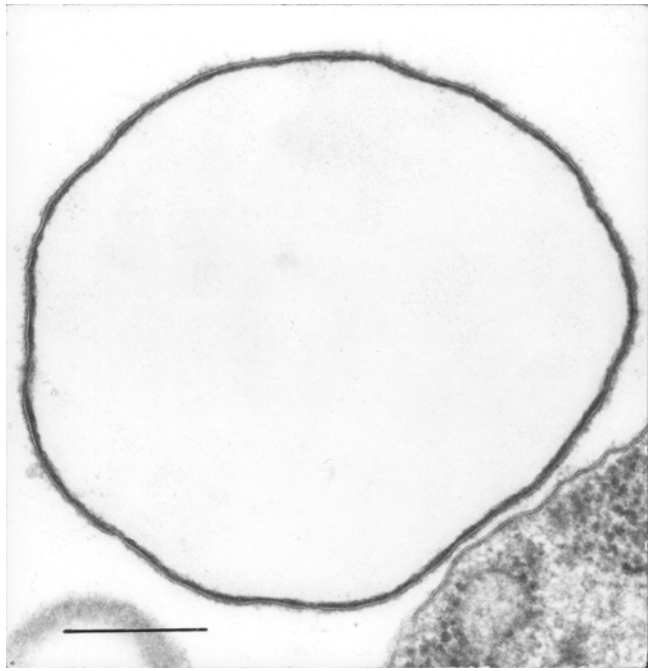

Figure 4.1. Electron micrograph of a milk fat globule showing the nature of the surrounding membrane. The membrane has a typical bilayer appearance and densely staining material coating the inner face. Bar = 0.1 μm. Reproduced from Keenan *et al.* (1988) with permission. Micrograph courtesy of W.W. Franke.

morphology and composition resemble droplets formed *in situ*. Using a proteomics approach to identify proteins associated with cytosolic lipid droplets, Wu *et al.* (2000) obtained evidence consistent with the interpretation that lipid droplets in milk originate from the endoplasmic reticulum.

Milk lipid globule precursors are present in the cytosol as droplets ranging in diameter from less than 0.5 to more than 4 μm (Dylewski *et al.*, 1984a; Deeney *et al.*, 1985). These precursors appear to originate as small droplets, with diameters under 0.5 μm, termed microlipid droplets. Droplets grow in volume by fusing with each other, giving rise to larger droplets, termed cytoplasmic lipid droplets (Figure 4.3). In a cell-free system, droplet fusion was promoted by calcium and by an, as yet, uncharacterized high molecular weight protein fraction from cytosol. Gangliosides appear to be involved in this fusion in some manner (Valivullah *et al.*, 1988). While small droplets fused readily, larger cytoplasmic lipid droplets did not fuse in the

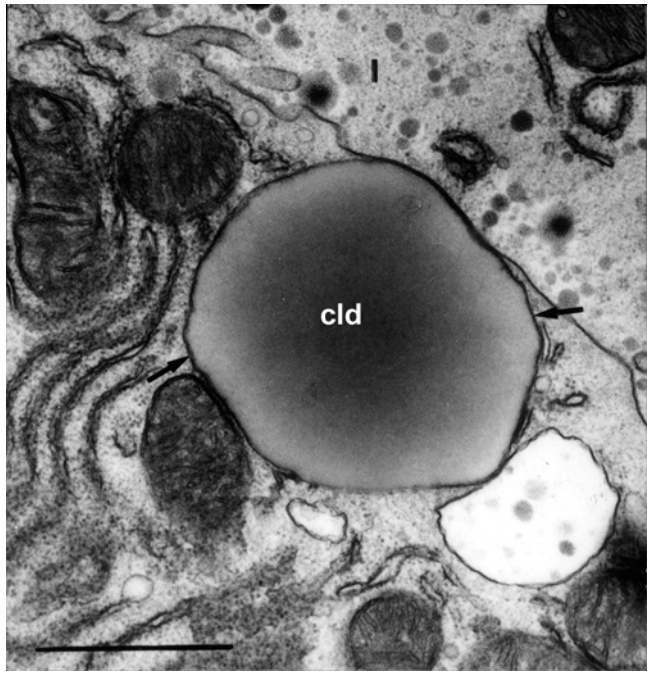

Figure 4.2. Electron micrograph showing a cytoplasmic lipid droplet (cld) in a cell fixed with potassium ferrocyanide. Dark-staining material of variable thickness coats the surface of the droplet (arrows). The alveolar lumen (l) is denoted. Bar = 1 μm. From Dylewski *et al.* (1984a) with permission.

cell-free system. The reasons for this are not apparent but may be related to some compositional differences between the coat material on micro-plasmic and cytoplasmic lipid droplets (Dylewski *et al.*, 1984a; Deeney *et al.*, 1985). Droplets of all sizes have a triacylglycerol-rich core surrounded by a protein and polar lipid coat. In composition, the protein and polar lipid material surrounding both micro-plasmic and cytoplasmic lipid droplets is similar but not identical.

While available evidence supports the view that an increase in the volume of lipid droplets occurs through fusion of lipid droplets, it is by no means certain that this is the only mechanism supporting droplet growth. Other possibilities include, e.g., growth *via* lipid transfer proteins that convey triacylglycerols from their site of synthesis to lipid droplets (Patton, 1973). Some 4% or more of the total lipid in lactating rat mammary gland is found in the cytosol and much of this complement of cytosolic lipid is

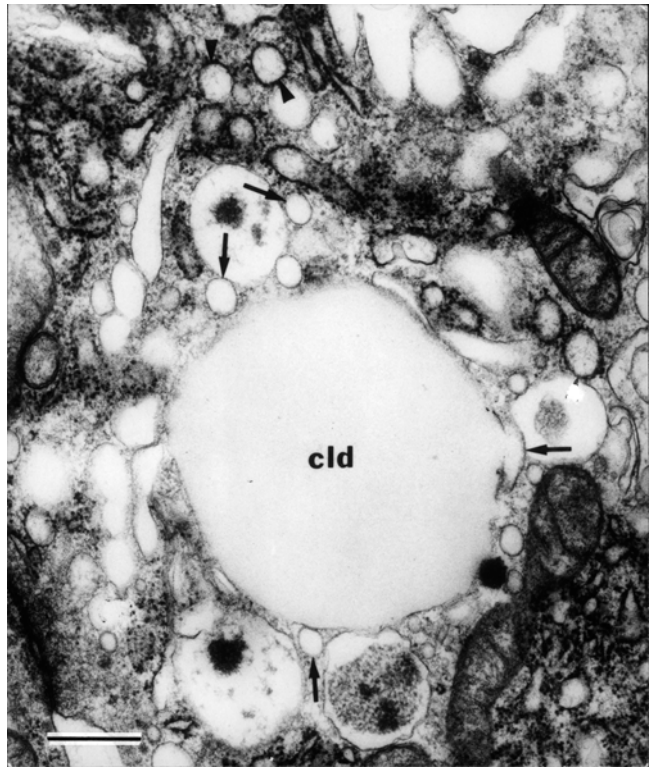

Figure 4.3. Electron micrograph showing a cytoplasmic lipid droplet (cld) and numerous microlipid droplets, some of which are denoted by arrows. An apparent fusion figure between the cld and a microlipid droplet is denoted by the arrow on the right. The specimen was fixed simultaneously with glutaraldehyde and osmium tetroxide. Bar = 0.5 μm.

associated with the fatty acid synthase complex (Keon *et al*., 1993). In a cell-free system, fatty acid synthase was able to transfer lipids to microlipid droplets and to the endoplasmic reticulum (Keon *et al*., 1993, 1994). Whether this transfer occurs in cells and supports droplet formation or growth remains to be determined. Further, whether droplet growth is purely random or is a regulated process is unknown. Monomeric GTP-binding proteins known to be involved in vesicular interactions, *arf, rab 3a*, and *rab 1a* or *b*, are associated with lipid droplets (Ghosal *et al*., 1993; T. W. Keenan, unpublished). This suggests the possibility that fusion may be regulated but this remains to be tested experimentally.

4.3. Intracellular Transit of Lipid Droplets

Lipid droplets migrate from their sites of origin, mostly in basal cell regions, to the apical pole of the cell, from whence they are secreted as milk lipid globules. Details of the guiding mechanism of this migration remain speculative but it is probable that cytoskeletal elements are involved. Droplets appear to transit exclusively toward the apical cytoplasm and bypass the supranuclear secretory cone formed by interconnected dictysomes of the Golgi apparatus (Dylewski *et al.*, 1984b). However, there is no morphological evidence for specific contacts between lipid droplets and elements of the cytoskeleton.

Microtubules are abundant in milk-secreting cells (Nickerson and Keenan, 1979) and, in guinea pigs, the tubulin content of mammary epithelial cells increases substantially during late pregnancy and the first half of lactation (Guerin and Loizzi, 1980). Attempts to interfere with microtubule assembly using colchicine and vinblastine have yielded contradictory results. Intramammary infusion of colchicine into lactating goats reversibly suppressed milk secretion (Patton, 1974). Colchicine did not appear to alter the rate of lipid synthesis in goats and, in treated glands, lipid droplets were larger than those in the contralateral untreated gland (Patton *et al.*, 1977). However, in the foregoing and in other studies, secretion of the serum phase of milk was suppressed also (Henderson and Peaker, 1980; Nickerson *et al.*, 1980). Addition of colchicine to tissue slices from lactating sheep and rabbits inhibited protein secretion but had no apparent effect on lipid secretion (Daudet *et al.*, 1981). In one study, intramammary infusion of colchicine into goats was found to suppress mammary extraction of constituents from serum and to suppress the rate of milk synthesis as well (Henderson and Peaker, 1980). Based on these, in part, disparate observations, and on the potential non-specific effects of colchicine and vinblastine (discussed in Mather and Keenan, 1983, 1998), we cannot ascribe a specific role to microtubules in milk fat secretion.

Actin-containing microfilaments are abundant in milk-secreting cells and are concentrated in apical cell regions (Amato and Loizzi, 1981). No actin staining was observed on luminal aspects in regions where fat droplets were in close apposition to the apical plasma membrane (Franke *et al.*, 1981). However, actin has been localized by immunofluorescence microscopy in the cytoplasmic surface along basal regions of budding lipid droplets. This observation raises the possibility that filamentous actin may form part of a contractile apparatus that functions in lipid secretion (Keenan *et al.*, 1988), a possibility that has yet to be investigated. Recently, and in contrast to a

previous finding (Keenan *et al.*, 1977), actin has been identified, immunologically, as a constituent of the MFGM (Heid and Keenan, 2005).

4.4. Secretion of Milk Fat Globules

Lipid droplets are secreted enveloped by cellular membranes. As first recognized by Bargmann and Knoop (1959), lipid droplets approach closely to, or contact, the apical surface and are gradually enveloped in membrane up to the point at which they dissociate from the cell, surrounded by plasma membrane (Figure 4.4; mechanism a, Figure 4.5). This is the widely-accepted mechanism of milk fat globule secretion (Patton and Keenan, 1975; Mather and Keenan, 1983, 1998; Keenan *et al.*, 1988; Keenan and Patton, 1995). Wooding (1971a, 1973) and others (Kralj and Pipan, 1992) obtained morphological evidence for an alternative mechanism based on an observed association between lipid droplets and secretory vesicles in apical cell regions

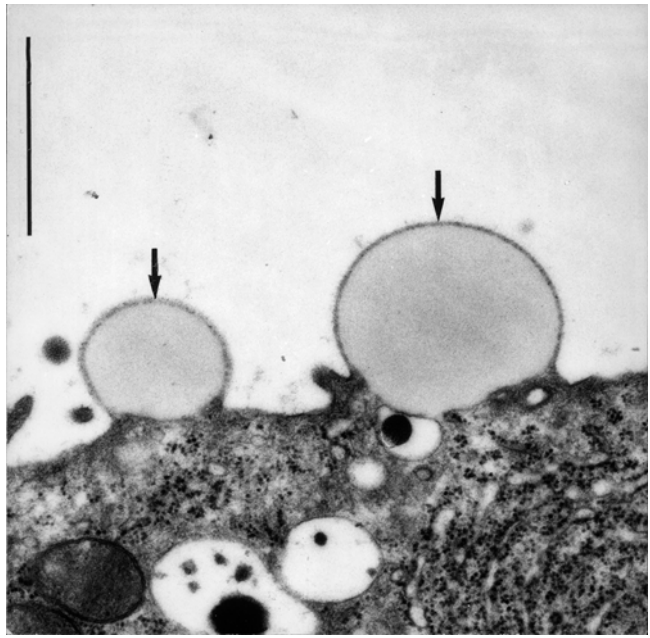

Figure 4.4. Electron micrograph showing two small lipid droplets (arrows) which, apparently, were in the process of being secreted. These droplets are partly enveloped in what appears to be plasma membrane in the apical cell region. Bar = 1 μm. From Deeney *et al.* (1985) with permission.

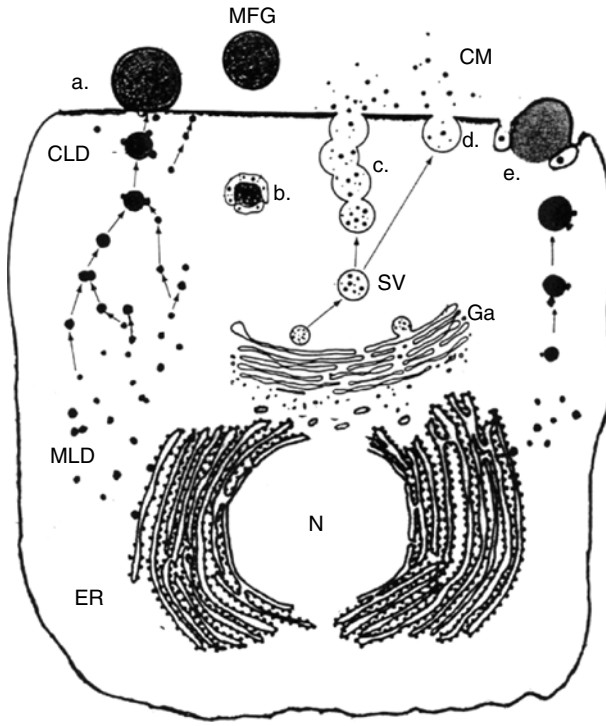

Figure 4.5. Diagrammatic summary of what is known about the intracellular origin, growth, and secretion of milk fat globules. (a) Secretion of a milk fat globule by envelopment in apical plasma membrane. (b) As originally proposed by Wooding (1971a), under certain conditions secretory vesicles may surround fat droplets and fuse together to form a vacuole containing the fat droplet. Presumably, these vacuoles are transported to the apical surface where the vacuolar contents are released by exocytosis. (c) Secretion of the serum (non-fat) phase of milk by compound exocytosis. (d) Secretion of the serum phase of milk by simple exocytosis. (e) A possible but as yet undocumented combination of apical plasma membrane and secretory vesicle membrane mechanisms for secretion of fat globules. Abbreviations: CLD, cytoplasmic lipid droplet; CM, casein micelle; ER, endoplasmic reticulum; Ga, Golgi apparatus; MLD, microlipid droplet; MFG, milk fat globule; N, nucleus; SV, secretory vesicle.

(see also Franke and Keenan, 1979; Stemberger *et al.*, 1984). Wooding (1971a, 1973) proposed that progressive fusion of neighboring vesicles on the surfaces of lipid droplets may lead to the formation of intra-cytoplasmic vacuoles containing both casein micelles and lipid droplets enveloped with secretory vesicle membrane (mechanism b, Figure 4.5). The content of such vacuoles may then be released from the cell by exocytosis. Wooding (1973) suggested that vacuole formation may occur *in vivo* at certain stages of the

secretory cycle, although these observations were made with specimens that may have been fixed for microscopic examination under less than optimal conditions.. Kralj and Pipan (1992) provided evidence suggesting that vacuolar lipid secretion may be common both during the parturient period and when milk secretion is inhibited temporarily.

By virtue of their static nature, electron micrographs provide no information on the kinetics of secretion. Just the process of fixation may provoke a temporary decrease in secretory events and a consequent accumulation of lipid droplets and secretory vesicles in cells before they succumb to the fixative agent. Such crowding in the apical cytoplasm could well lead to promiscuous associations and interactions that are unrelated to normal secretory processes (Mather and Keenan, 1998).

Contributions to the MFGM from the apical plasma membrane and secretory vesicle membrane could be assessed by direct biochemical comparisons of these membranes isolated from mammary homogenates. However, technical challenges inherent to the isolation of secretory vesicle and apical plasma membrane have yet to be overcome. Well-characterized apical plasma membrane preparations from mammary gland have not been forthcoming; preparations of plasma membranes from this source are enriched in baso-lateral membrane fragments and may contain plasma membranes from other cell types (Keenan *et al*., 1970, 1989). Nevertheless, comparisons of plasma membranes isolated from mammary gland with MFGM show these membranes to be similar in polar lipid composition and some protein constituents (Keenan *et al*., 1970, 1989). Secretory vesicles from the mammary gland are osmotically fragile and have proven difficult to isolate. Preparations that have been described differ substantially from the MFGM (Sasaki *et al*., 1978; Keenan *et al*., 1979). However, these preparations appear to be enriched in immature vesicle membranes, which may have a different composition from the membranes of mature secretory vesicles. Based on these considerations, no realistic interpretations about the plasma membrane or secretory vesicle membrane origin of MFGM can be made from the extant biochemical data (Mather and Keenan, 1998).

Available cytochemical evidence favors plasma membrane envelopment as the principal mechanism for the secretion of milk fat globules. Butyrophilin (BTN), a major integral membrane protein of milk fat globules, is expressed on the apical plasma membrane (Franke *et al*., 1981) and appears to be concentrated in areas of the membrane associated with budding lipid droplets (Mather, 1987). By immunoperoxidase labeling, BTN was not detected on intracellular membranes (Franke *et al*., 1981). The MUC1 mucin also is concentrated in apical regions of the plasma membrane and in MFGM (Johnson and Mather, 1985; Mather *et al*., 2001). In contrast, secretory vesicle membranes contain only about 10% of the amount of MUC1 estimated, by

immunogold labelling, to be associated with apical plasma membrane and the MFGM. These observations make it unlikely that substantial portions of the MFGM originate from secretory vesicle membranes. Finally, more than 80% of the total number of fat globules in cows' milk are less that 1 μm in diameter (Mulder and Walstra, 1974), a size incompatible with the direct involvement of mature secretory vesicles in the secretion of these small globules. Morphological evidence suggests that these small globules are secreted by envelopment with plasma membrane (Figure 4.4).

During the budding of lipid droplets from the apical surface, a uniform distance of 10 to 20 nm is maintained between the outer surface of the droplet and the cytoplasmic face of the plasma membrane (Wooding, 1971a). This intervening space appears in micrographs to be filled with an electron-dense material that may originate from the cytoplasm or from the inner face of the apical plasma membrane. During secretion, this material is most likely carried over into milk fat globules and gives rise to the characteristic coat visible as a fuzzy, amorphous layer on the inner face of the MFGM isolated from cream (Wooding and Kemp, 1975; Freudenstein *et al.*, 1979) (Figure 4.6). The origin and nature of this material was the subject of much speculation, primarily because understanding its origin and nature appeared to be a key towards understanding the mechanism for milk fat

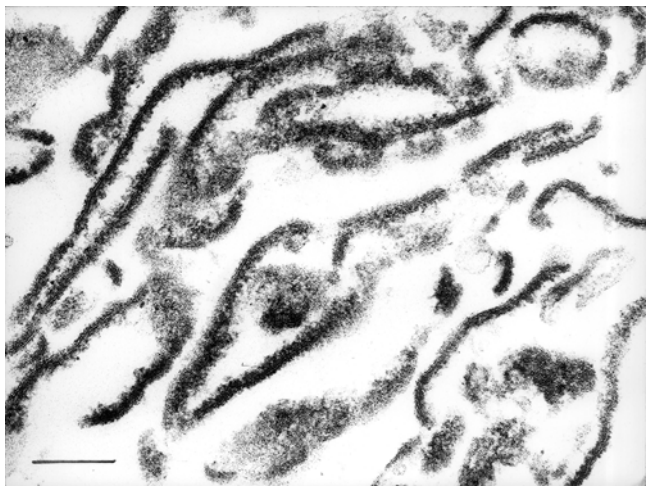

Figure 4.6. Electron micrograph of MFGMs which were released from washed milk fat globules by churning and collected by ultracentrifugation. These membranes occur mostly as sheets and show little tendency to vesiculate. Membranes retain the dense-staining protein coat material that originally was oriented toward the globule interior. Bar = 0.2 μm.

globule secretion. Studies using various extractants established that the MFGM coat was primarily proteinaceous in nature (Wooding and Kemp, 1975; Freudenstein *et al*., 1979) and freeze-etch micrographs have shown that it exists in a paracrystalline hexagonal array with long-range order (Wooding, 1977; Buchheim, 1982).

Recent work on the characterization of proteins of the MFGM has done much to reveal the nature of this coat material. From a consideration of protein topologies and protein-protein interactions, it is likely that the protein coat comprises the cytoplasmic tail of BTN, a type 1 transmembrane glycoprotein, the peripheral protein xanthine dehydrogenase/oxidoreductase (XDH), and the intracellular lipid-droplet-associated protein adipophilin (ADPH, known also as adipocyte differentiation-related protein or ADRP) (for reviews, see Mather and Keenan, 1998; Mather, 2000). There is substantial evidence that BTN and XDH interact directly with each other. In cows, these two proteins are expressed in constant molar proportions throughout lactation (Mondy and Keenan 1993) and they can be covalently bound to each other by bifunctional cross-linking reagents (Valivullah and Keenan, 1989). The cytoplasmic tail of BTN, expressed as a recombinant fusion protein in *E. coli*, specifically binds to XDH *in vitro* (Ishii *et al*., 1995). XDH can be released from the MFGM by the reduction of disulfide bonds (Spitzberg and Gorewit, 1998) and proteins in the detergent-insoluble membrane coat complex can be dissociated by exogenous thiol reagents (I.H. Mather and T.W. Keenan, unpublished). This suggests that interactions between XDH and BTN are promoted, or stabilized, by disulfide bonds.

Direct evidence that XDH and BTN are essential for normal milk lipid secretion has recently been provided by the use of gene-targeting techniques (Vorbach *et al*., 2002; Ogg *et al*., 2004). Heterozygous mice with one ablated XDH allele ($Xdh^{+/-}$) displayed deficiencies in lipid secretion with consequent accumulation of lipid within mammary epithelial cells. The milk of these animals is characterized by the presence of large lipid droplets with disrupted membranes. Lactation in mice with both XDH alleles ablated ($Xdh^{-/-}$) could not be studied because such mice did not survive beyond six weeks of age (Vorbach *et al*., 2002). The same phenotype was observed in mice in which BTN expression was either disrupted or eliminated (Ogg *et al*., 2004). We propose that interactions between a BTN/XDH complex and other proteins on the intracellular lipid droplet surface mediate interactions between lipid droplets and membrane (Mather and Keenan, 1998). Since XDH is a homodimer, binding to BTN may initially induce the formation of BTN dimers which could further aggregate through adhesive interactions to form large complexes of the type observed in freeze-etch micrographs. Alternatively, BTN may first self-aggregate in the plane of the membrane

bilayer and bind to XDH homodimers (A. Rao and I.H. Mather, unpublished observations). Such protein-protein interactions could well explain the progressive envelopment of lipid droplets by plasma membrane, although this remains speculative. Disulfide bond formation also may play a role, since protein disulfide isomerase has been identified as a constituent protein of intracellular lipid droplets (Ghosal *et al*., 1994; Wu *et al*., 2000) and, as discussed above, XDH can be released from the MFGM by thiol reagents.

That other proteins are associated with the MFGM coat is probable, particularly proteins associated with the surface of intracellular lipid droplets. However, several of the proteins identified as being associated with intracellular lipid droplets (Wu *et al*., 2000) have yet to be identified as constituents of the MFGM coat. Two proteins associated with intracellular lipid droplets, protein disulfide isomerase (Ghosal *et al*., 1994) and the nuclear coactivator protein p100 (Keenan *et al*., 2000) are absent from MFGM preparations. Thus, there apparently is some selectivity in which of the proteins associated with intracellular lipid droplets are secreted.

The mechanisms controlling milk fat secretion remain unknown. Protein kinases may be implicated because the release of lipid from primary cultures of rat mammary epithelial cells was stimulated by protein kinases (Rohlfs *et al*., 1993; Spitsberg and Gorewit, 1997) or phosphatases (review, Keenan *et al*., 1988) are associated with MFGM preparations. CD36, a component of the MFGM, may associate with src family protein kinases in a number of cell types and play some role in intracellular signalling (Greenwalt *et al*., 1992). That monomeric GTP-binding proteins of the type known to be involved in vesicle-membrane interactions are associated with intracellular lipid droplets and the MFGM suggests a potential regulatory function for G-proteins in milk fat secretion, particularly since a non-hydrolyzable analogue of GTP was shown to stimulate fat secretion from permeabilized rat mammary acini (Ghosal *et al*., 1993). However, due to lack of any specific information, the control or regulation of milk fat secretion remains an area purely of speculation. What currently is known about the origin, intracellular growth, and secretion of milk fat globules is summarized in Figure 4.5.

During the secretion process, milk fat globules usually are enveloped compactly by membrane but in some instances closure of the membrane entrains some cytoplasm between the membrane and the fat droplet surface. The result is an extracellular fat globule with a so-called signet or crescent of cytoplasm attached (Figure 4.7). These crescents can vary from thin slivers of cellular material to large inclusions that exceed the volume of the fat globule core. Wooding (1977), in his alternative scheme for fat globule secretion, suggested that crescents originate from the trapping of cytoplasm as secretory vesicles fuse around the droplets during vacuole formation.

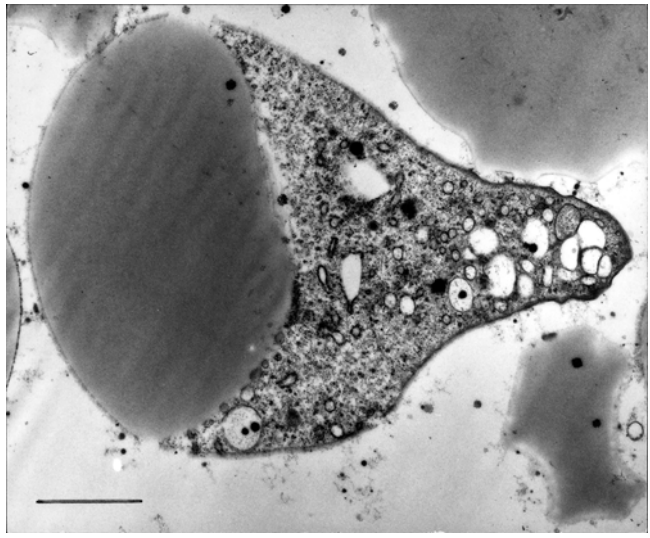

Figure 4.7. Electron micrograph of a milk fat globule in the alveolar lumen that has a large cytoplasmic crescent entrained between the globule and the surrounding membrane. This crescent contains secretory vesicles, ribosome-studded vesicles of apparent endoplasmic reticulum origin, and an abundant amount of particulate material. Bar = 2 μm.

Huston and Patton (1990) suggested that an abnormality in the protein coat along the cytoplasmic face of the apical plasma membrane may be responsible for the phenomenon of crescent formation. Inadequate amounts of proteins forming this coat or abnormalities in the distribution of the coat complex may interfere with adhesion of the membrane to the droplet.

Crescents have been observed, morphologically, to contain nearly all intracellular membranes and organelles of the milk-secreting cell, except nuclei. The proportion of globules with crescents appears to vary both between species and in individuals within a species. Between 1 to 5% of the globules in goat and guinea pig milks and about 1% of the globules in cows' milk were observed to contain crescents (Wooding *et al.*, 1970; Huston and Patton, 1990). Janssen and Walstra (1982) measured the quantity of cresent material in the milk of cows, goats, rats, pigs, sheep, rabbits, and humans and found that cows have the lowest, and rabbits have the highest, quantity of fat globule-associated crescent material. In humans, Patton and Huston (1988) found a diurnal variation in crescent frequency.

Gaining an understanding of the molecular mechanism of crescent formation would advance our understanding of the process of milk fat globule secretion. Crescents are a route by which cellular constituents can

enter milk. This may be a route for the entry of bioactive molecules such as hormones or growth factors into milk.

4.5. Isolation and Gross Composition of MFGM

Some of the membrane that initially surrounds fat globules is lost following secretion, either within the mammary gland or in expressed milk (Wooding, 1971b, 1974). Estimates of the extent of this loss vary considerably (Mather and Keenan, 1983). That changes occur also in MFGM after secretion is probable, but there are few quantitative data available on these changes. Clearly, the manner in which milk is collected and handled also may cause loss and compositional alteration of the MFGM, i.e., the nature of the MFGM as isolated and studied may not necessarily reflect the membrane as it was when fat globules were secreted initially (for review, see Evers, 2004).

Membranes can be released from fat globule suspensions by several methods, including churning (agitation), freezing and thawing, treatment with detergents, or by suspension in polar, aprotic solvents. Once released, MFGM can be collected readily by centrifugation at a high centrifugal force or, after inducing membrane aggregation, at a lower force. Details of these methods and the effects of the preparative method on the composition of the resultant MFGM have been reviewed and will not be repeated here (Keenan *et al.*, 1988; Keenan and Dylewski, 1995).

Information on the gross composition of MFGM is summarized in Table 4.1. Proteins and lipids account for 90%, or more, of the dry weight of the MFGM. However, reported amounts of the total membrane weight

Table 4.1. Gross composition of bovine MFGM[a]

Constituent class	Unit	Amount
Protein	weight %	25–60
Total lipids	mg/mg protein	0.5–1.1
Phospholipids	mg/mg protein	0.13–0.34
Neutral lipids	mg/mg protein	0.25–0.88
Glycosphingolipids	μg/mg protein	13[b]
Hexoses	μg/mg protein	108
Hexosamines	μg/mg protein	66
Sialic acids	μg/mg protein	20
Glycosaminoglycans	μg/mg protein	0.1
RNA	μg/mg protein	20

[a] From data compiled and reviewed in Keenan *et al.* (1988) and Keenan and Dylewski (1995).
[b] Calculated assuming average molecular weights of neutral glycosphingolipids and gangliosides of 850 and 1470 Da, respectively.

accounted for by proteins and specific lipids vary considerably. In particular, there is wide variation in reported values for neutral lipid, generally measured so as to include sterols and steryl esters, unesterified fatty acids, and monoacylglycerols, diacylglycerols and triacylglycerols. Much of this reported variation is undoubtedly due to methodological differences in membrane preparation. Hexoses, hexosamines and sialic acids in the MFGM appear to be bound exclusively covalently to proteins and glycophingolipids. MFGM, like plasma membrane, is enriched in glycosphingolipids.

Several workers have detected RNA in MFGM preparations. Swope and Brunner (1965) estimated that bovine MFGM contains about 20 μg of RNA per mg protein. Jarasch *et al.* (1977) measured about the same amount of RNA but were able to reduce this amount to about 10 μg/mg protein by extraction of MFGM with high ionic strength buffers. This RNA may originate from ribosomes associated with the surface of lipid droplets within the cell (Dylewski *et al.*, 1984a), or it may be from fragments of endoplasmic reticulum or ribosomes trapped in cytoplasmic crescents. DNA has not been found in MFGM preparations (Jarasch *et al.*, 1977). Why glycosaminoglycans, normal basement membrane constituents, are found in the MFGM remains to be explained. Hyaluronic acid, chondroitin sulfate and heparin sulfate have been identified as constituents of glycosaminoglycan fractions from MFGM (Lis and Monis, 1978; Shimizu *et al.*, 1981).

4.6. Lipid Composition of the MFGM

Triacylglycerols usually are the most abundant lipid class found in MFGM (Table 4.2) but the preparative method has a major influence on the amount in individual preparations, probably because variable quantities of triacylglycerols remain associated with the membrane during isolation. MFGM-associated triacylglycerols contain higher proportions of palmitate and stearate (i.e., long-chain fatty acids with relatively high melting points) than do triacylglycerols of the core fat. However, MFGM was not enriched in high-melting point triacylglycerols when membranes were isolated from fat globules destabilized at an elevated temperature (Vasic and DeMan, 1966; Bracco *et al.*, 1972). Walstra (1974) suggested that high-melting point triacylglycerols originate from fat crystals that adhere to the membrane during cooling and churning. Most triacylglycerols are assumed to be associated with the cytoplasmic face of the MFGM, i.e., with the region in contact with the globule core; the outer face of milk fat globules appears to contain little neutral lipid (Newman and Harrison, 1973).

Monoacylglycerols and diacylglycerols may be genuine membrane constituents or may be products of lipolysis. Unesterified fatty acid levels

Table 4.2. Lipid composition of bovine MFGM[a]

Constituent class	Proportion of total lipids (%)
Triacylglycerols	62
Diacylglycerols	9
Monoacylglycerols	Traces
Sterols	0.2–2
Sterol esters	0.1–0.3
Unesterified fatty acids	0.6–6
Hydrocarbons	1.2
Phospholipids	26–31
Constituent class	Proportion of total phospholipids (%)
Sphingomyelin	22
Phosphatidylcholine	36
Phosphatidylethanolamine	27
Phosphatidylinositol	11
Phosphatidylserine	4
Lysophosphatidylcholine	2

[a] From data compiled in Patton and Keenan (1975), Keenan *et al.* (1988) and Dylewski and Keenan (1995).

in the MFGM vary widely between preparations and, again, they may originate by lipolysis. There is extensive variation in the amount of sterols, principally cholesterol, reported to occur in the MFGM. Given the probable plasma membrane origin of the primary MFGM and the fact that plasma membranes are enriched in cholesterol (van Meer, 1989), it is not surprising that the MFGM contains abundant cholesterol. Cholesterol accounts for over 90% of the total sterols in cows' milk (Blanc, 1981); other sterols have been identified but it is not clear whether they are components of the MFGM.

Of the total phospholipid pool in milk, about 60% occurs in the MFGM; the remainder is found primarily associated with the membrane fraction in skim milk (Huang and Kuksis, 1967; Patton and Keenan, 1971). Phospholipids of the fat globule are recovered nearly quantitatively with the MFGM when globules are destabilized at 40°C. The same five major phospholipids present in bovine MFGM (Table 4.2), are found also in the milk or MFGM from several other species with a similar pattern of distribution (for review, see Keenan and Dylewski, 1995). The phospholipid distribution pattern of the MFGM is similar to that of plasma membranes from mammary gland in that the sphingomyelin to phosphatidylcholine ratio is higher than that of intracellular membranes (Keenan *et al.*, 1970, 1988; Kanno, 1990). Lyso-derivatives of phosphatidylcholine and phosphatidylethanolamine are found in the MFGM but in minor amounts in samples handled so as to minimize lipolysis. The proportions of the various phosphoglyceride

classes present in the MFGM as alkyl or alkenyl ethers has not been determined.

The distribution and fatty acid composition of phospholipids in skim milk are similar to those in the MFGM. This has led to the suggestion that these membrane-associated constituents originate from a common cellular source and that the skim milk membranes, which contain much of the skim milk phospholipid pool, may be shed MFGM (for review, see Keenan and Dylewski, 1995). This assumption remains to be tested critically.

Glycosphingolipids are relatively minor constituents of bovine MFGM (Table 4.1) but they have been studied widely because of the known roles of glycosphingolipids and some of their breakdown products in a number of biological phenomena such as growth regulation through modulation of protein kinases and phosphatases (Merrill, 2002; Smith and Merrill, 2002). Bovine MFGM contains two neutral glycosphingolipids, glycosyl-ceramides and lactosyl-ceramides, in nearly equimolar proportions. Neutral glycosphingolipids with more complex carbohydrate chains have not been detected. Nine gangliosides (glycosphingolipids containing at least one molecule of sialic acid) in bovine MFGM have been characterized structurally. The two major gangliosides in bovine MFGM are GD3 and GM3 (nomenclature of Svennerholm, 1963); the other seven gangliosides, in total, account for about 20% of the ganglioside content of the MFGM (for reviews, see Jensen and Newburg, 1995; Keenan and Patton, 1995).

4.7. Enzymes Associated with the MFGM

To date, about 28 different enzymes or enzymatic activities have been detected in MFGM preparations from cows' milk (Table 4.3). Since the compilation in the previous edition of this book (Keenan and Dylewski, 1995), protein kinase activity that phosphorylates some MFGM proteins has been identified in MFGM preparations (Spitsberg and Gorewit, 1997). The NADH oxidase of MFGM, originally identified by its ability to reduce cytochrome c and ferricyanide (Jarasch *et al.*, 1977; Bruder *et al.*, 1978, 1982), was recently shown to have an activity that oscillates with a period of 24 min (Morré *et al.*, 2002).

Some of the enzymes of the MFGM, such as 5′-nucleotidase, adenosine triphosphatase and phosphodiesterase I, are known to be enriched in plasma membranes. However, other enzymes found in the MFGM are known constituents of intracellular membranes or are cytosolic. Why some of these are present in the MFGM remains to be explained; some may possibly originate from material entrained in cytoplasmic crescents and therefore are not true MFGM constituents. Perhaps some enzymes become

Table 4.3. Enzymatic activities found in bovine MFGM[a]

Enzymatic activity[b]	EC number
Xanthine oxidoreductase	1.2.3.2
Lipoamide dehydrogenase	1.6.4.3
NADPH oxidase	1.6.99.1
NADH oxidase	1.6.99.3
Sulfhydryl oxidase	1.8.3.2
Catalase	1.11.1.6
γ-Glutamyl transpeptidase	2.3.2.1
Galactosyl transferase	2.4.1._
Protein kinase(s)[c]	2.7.1._
Cholinesterase	3.1.1.8
Alkaline phosphatase	3.1.3.1
Acid phosphatase	3.1.3.2
Phosphatidic acid phosphatase	3.1.3.4
5′-Nucleotidase	3.1.3.5
Glucose-6-phosphatase	3.1.3.9
Phosphodiesterase I	3.1.4.1
UDP-glycosyl hydrolases	3.2.1
β-Glucosidase	3.2.1.21
β-Galactosidase	3.2.1.23
Hexosaminidase[d]	3.2.1.52
Plasmin	3.4.21.7
Inorganic pyrophosphatase	3.6.1.1
Adenosine triphosphatase	3.6.1.3
Thiamine pyrophosphatase[e]	3.6.1.6
Nucleotide pyrophosphatase	3.6.1.9
Aldolase	4.1.2.13
Acetyl-CoA carboxylase[f]	6.4.1.2

[a] Primary references are compiled in Keenan *et al.* (1988), Keenan and Dylewski, (1995) and Mather (2000).
[b] Enzymes are listed by their common names.
[c] Spitsberg and Gorewit (1997).
[d] Kitchen *et al.* (1978).
[e] Sasaki *et al.* (1978).
[f] Enzymatically inactive (Shriver *et al.*, 1989).

adsorbed on the surface of lipid droplets intracellularly and are thus fortuitously associated with secreted fat globules. Several of the enzymes associated with the MFGM have been purified and at least partially characterized; this information has been reviewed by Keenan *et al.* (1988).

Studies on the functional significance of MFGM-associated enzymes have been restricted largely to their involvement in degradative events that affect processing properties or flavor, or for their use as markers of adequate pasteurization. Obviously, enzymes that can produce product defects are of great concern to the dairy industry. Unfortunately, the biological roles

in fat globule formation or secretion, if any, of MFGM-associated enzymes are unknown.

4.8. Proteins of the MFGM

This area of MFGM research has advanced considerably since the previous edition of this book (Keenan and Dylewski, 1995). Some heretofore unrecognized MFGM proteins have been identified, the sequences of the major proteins have been determined, mostly *via* cDNA sequencing, and a function has been ascribed to some proteins. Much of the knowledge in this area has been reviewed extensively (Mather, 2000). Herein, we will provide a summary of this information but will not include sequences. Appropriate databank accession numbers are given for those MFGM proteins that have been sequenced. The major proteins of bovine MFGM, for which the sequence has been determined, are the epithelial mucins MUC1 and MUC15, Xanthine oxidoreductase (XDH), cluster of differentiation 36 (CD36), butyrophilin (BTN), adipophilin (ADPH; this protein is known also as adipocyte differentiation-related protein), periodic acid Schiff glycoprotein 6/7 (PAS6/7), and fatty acid-binding protein (FABP) (Figure 4.8). The nomenclature used herein will be largely that recommended by Mather (2000). This latter article also lists other names used for the major MFGM proteins.

The MFGM of cow, and of most other ruminants, rodent and primate species that have been analyzed, contain one or more heavily-glycosylated mucin-like glycoproteins which stain well with the PAS reagent, variably with a modified silver stain, but poorly or not at all with Coomassie blue. Bovine MFGM contains at least two mucins, MUC1 and MUC15 (formerly PAS III), which have been characterized by biochemical and molecular cloning techniques.

MUC1 mucins have type 1 topologies (i.e., an externally oriented N-terminus and a single transmembrane domain), extensively glycosylated exoplasmic domains, a variable number of tandem repeats in the amino acid sequence, and a short cytoplasmic tail (reviews, Patton *et al*., 1995; Mather, 2000) (Figure 4.9). During sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), MUC1 migrates with a slower mobility than XDH. In mixed herd milk, MUC1 is seen as a diffuse, smeared band due to allelic polymorphism, although in milk from individual animals it can be resolved as one or two discrete bands. Five alleles have been identified in Holstein cattle in the United States, with an apparent molecular weight ranging from about 160 to 200 kDa (Hens *et al*., 1995; Huott *et al*., 1995). Several MUC1 alleles have been identified in other breeds of cattle (for review; see Mather, 2000).

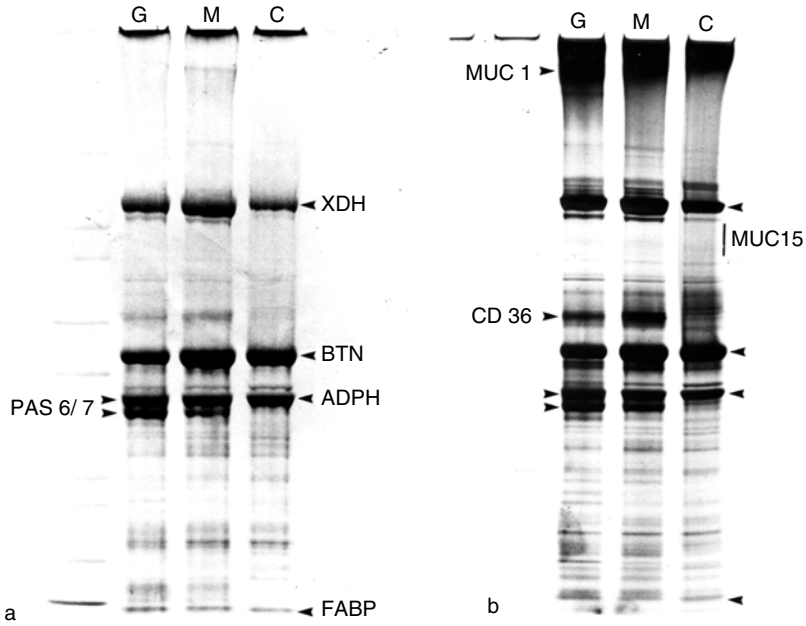

Figure 4.8. Polypeptides associated with washed milk fat globules (G), released milk fat globule membranes (M), and with the detergent and high salt-insoluble MFGM coat material. (a) Gel was stained with Coomassie blue. The abbreviations are XDH, xanthine dehydrogenase/oxidoreductase; BTN, butyrophilin; ADPH, adipophilin; PAS 6/7, periodic acid Schiff 6/7; FABP, fatty acid binding protein. (b) Gel was stained with silver. The positions of the major proteins, including MUC1 and CD36, are indicated by arrowheads and the approximate position of MUC15 by a bar (MUC15 is best detected with the PAS reagent, see Mather, 2000). The unlabeled arrowheads in (b) denote the corresponding proteins identified in (a). SDS-PAGE was in 8 to 16% Tris-glycine gels.

Bovine milk MUC1 is present in both cream and skim milk membranes, at an estimated total concentration of up to about 40 mg/L of milk (Mather, 2000). MUC1 has been purified from bovine MFGM and a full-length cDNA described (**EMBL accession number AJ400824**; Pallesen *et al.*, 2001). The complete sequence is available also for MUC1 from human (**J05581**), gibbon (**L41589**), mouse (**M65132**) and other species. While the amino acid sequence predicts a trans-membrane protein, milk MUC1 behaves anomously as a soluble protein and can be displaced readily, at least in part, from the MFGM (Mather, 2000). Soluble forms of MUC1, lacking the membrane anchor, have been described. In cultured cells, a proteolytic cleavage occurs in the exoplasmic domain, generating a noncovalently associated heterodimer (Ligtenberg *et al.*, 1992). There is some preliminary

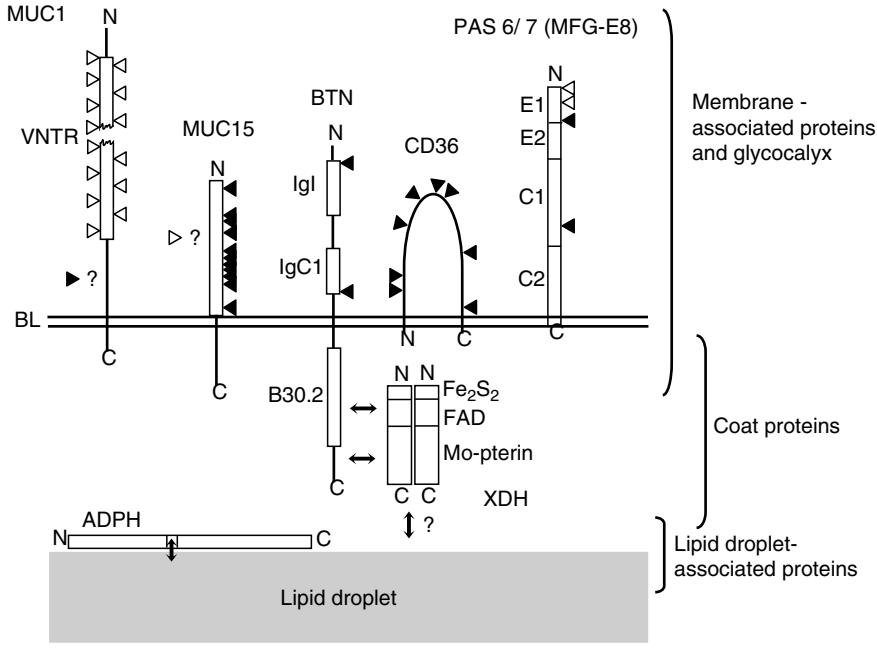

Figure 4.9. Protein topology and proposed structure of the bovine MFGM. Proteins and their constituent domains are drawn schematically showing the approximate positions of N-linked glycans (closed triangles) and O-linked glycans (open triangles). The number and positions of N-linked glycans associated with MUC1, and of O-linked glycans associated with MUC15 is uncertain. Glycosylation sites in PAS6/7 are shown as a composite of both PAS6 and 7. Integral proteins of the bilayer (BL) include MUC1 with associated variable-number-tandem repeats (VNTRs) (indicated as a break in the sequence), MUC15, CD36 and BTN with associated intermediate (IgI) and constant (IgC1) Ig domains in the exoplasmic domain and B30.2 region in the cytoplasmic domain. PAS6/7 with associated EGF-like repeats (E1 and E2) and factor-V- and VIII-like domains (C1 and C2) may be anchored to the membrane *via* a C-terminal amphipathic alpha-helical domain. Glycans associated with MUC1, MUC15, BTN, CD36 and PAS6/7 and additional glycosaminoglycans (not shown) contribute to the outer glycocalyx of the membrane. The major constituents of the protein coat on the inner surface of the MFGM comprise XDH [shown as a homodimer, with constituent Fe/sulfur clusters (Fe_2S_2), flavin-binding domain (FAD) and molybdo-pterin domain (Mo-pterin)], the cytoplasmic tail of BTN and ADPH. ADPH is bound to the lipid droplet surface through a presumptive alpha-helical domain. Double arrowheads indicate interactions between BTN and XDH and between ADPH and the lipid droplet surface discussed in the text. Whether XDH also interacts with the lipid droplet surface is speculative. In molar terms, BTN and XDH are the most abundant membrane-associated proteins. Figure adapted from Figure 4.5b of Mather and Keenan (1998).

evidence that such is the case with bovine MFGM MUC1 (S. Patton and T. W. Keenan, unpublished).

Apparently MUC1 does not play a direct role in milk-fat globule secretion, as MUC1 knock-out mice lactate normally and raise litters successfully (Spicer *et al.*, 1995). This does not preclude an ancillary role for MUC1 in the secretory process or a role in stabilizing the MFGM. There is some evidence that MUC1 plays an immuno-protective role in the suckling neonate by sequestering pathogenic microorganisms (for reviews, see Mather, 2000; Patton, 2001; Peterson *et al.*, 2001).

A second mucin, MUC15, previously called PAS III, in bovine MFGM (Kaetzel *et al.*, 1987; Mather, 2000), has been isolated recently and characterized by biochemical and molecular cloning techniques (**EMBL accession number AJ417816**; Pallesen *et al.*, 2002). The human homologue also was cloned and shares 67% similarity in linear sequence with bovine MUC15. MUC15 was identified originally as a diffuse band of PAS-reactive material with an apparent molecular weight between about 95 to over 100 kDa, following SDS-PAGE (see, for example, Mather, 2000). Typically, this glycoprotein does not stain with Coomassie blue and variably with the silver reagent (position shown by a square bracket in Figure 4.8).

Like MUC1, MUC15 is a type 1 glycoprotein with an extensive exoplasmic domain, a single membrane span and a short cytoplasmic tail (Figure 4.9). The predicted molecular mass of 33.3 kDa is much smaller than expected from the electrophoretic mobility in SDS-polyacrylamide gels, suggesting that a large proportion of the mass of MUC15 is carbohydrate (Pallesen *et al.*, 2002). Both O-linked and N-linked glycans were detected by biochemical methods. Unlike MUC1, there are no tandem repeats in MUC15. The polydisperse character of MUC15 is, therefore, presumably due to the addition of variable amounts of carbohydrate. A splice variant, encoding a soluble form of MUC15, lacking the transmembrane domain (MUC15/S), was described also. MUC15 and MUC15/S, are widely expressed (at least at the RNA level) in many epithelial and non-epithelial cells and are currently of unknown function.

XDH is a major protein of the MFGM, with a monomeric molecular mass of about 155 kDa, estimated by SDS-PAGE (Figure 4.8). The active form of this enzyme is a homodimer. XDH accounts for about 20% of the globule-associated protein (Mather *et al.*, 1977). Upon disruption of the membrane, large amounts of XDH are recovered in the MFGM supernatant, and the remainder is bound tightly to the membrane (Mather *et al.*, 1977; Jarasch *et al.*, 1981). This bound XDH is recovered largely in the high salt and non-ionic detergent-insoluble MFGM coat (Freudenstein *et al.*, 1979). XDH is a widely-studied redox enzyme and has been the subject of numerous reviews (for references, see Mather, 2000). The sequence of XDH

from several species has been determined; in most cases from the cDNA. The sequence of bovine XDH (**Genbank accession number X83508**) is very similar to that of several other mammalian species. The amino acid sequence identity between bovine, rat, mouse, and human enzymes is close to 90% (Mather, 2000).

While XDH plays a key role in purine catabolism, the large amount of this enzyme associated with fat globules suggests a function beyond purine oxidation. As discussed above, XDH may play a role in milk fat globule secretion. Expression of the XDH gene increases during pregnancy and becomes maximal after parturition (McManaman *et al*., 2002). Recently, direct evidence for a role for XDH in milk fat secretion was obtained. Vorbach *et al*. (2002) generated mice with a targeted disruption in the (*Xdh*) gene. ($Xdh^{-/-}$) mice were runted and did not live beyond 6 weeks. ($Xdh^{+/-}$) females were healthy and fertile but were unable to maintain lactation; in these animals, the mammary epithelium collapsed, resulting in premature involution. XDH was found to be necessary for enveloping lipid droplets in plasma membrane during secretion. In ($Xdh^{+/-}$) mice, mammary epithelial cells became engorged with lipid and secreted lipid globules had a fragmented outer membrane that caused them to aggregate into very large droplets.

Cluster of differentiation 36 (CD36), a protein of 76 to 78 kDa, stains weakly with Coomassie blue but strongly with the PAS reagent and modified silver stain (Figure 4.8). This integral protein of the MFGM is heavily glycosylated. The complete amino acid sequence derived from the cDNA from cloned bovine mammary gland is available (**Genbank accession number X91503**). CD36 is known to have a number of diverse, mostly receptor, functions in the vascular and hemopoietic systems, but none of these functions is such as to suggest any role in milk fat secretion (Mather, 2000). However, there is evidence that human CD36 can associate with *src*-family kinases and function in intracellular signalling (Greenwalt *et al*., 1992). Given the evidence suggestive of the involvement of kinases/phosphatases in milk-fat secretion (discussed in the section on milk fat-globule secretion), the possible role of CD36 as a modulator of lipid secretion should be explored.

BTN is the most abundant protein of bovine MFGM and has been estimated to account for about 35 to 40% of the total MFGM protein in Holstein milk and for about 20% of MFGM protein in Jersey milk (Mather *et al*., 1980; Mondy and Keenan, 1993). The name "butyrophilin" is derived from the Greek *butyros* and *philos* meaning an affinity for butterfat (Franke *et al*., 1981). BTN migrates during SDS-PAGE with an estimated molecular mass of about 66 kDa and stains readily with Coomassie blue and silver (Figure 4.8). The exoplasmic domain contains two N-linked glycans that have been characterized extensively (Sato *et al*., 1995; Mather, 2000). BTN is

expressed specifically in the mammary gland and is concentrated in the apical plasma membrane and the MFGM. On destabilization of fat globules, most of the BTN remains associated with the membrane. Upon disruption of membrane structure by extraction with chaotropic agents, high salt or non-ionic detergents, much of the BTN remains associated with the insoluble MFGM coat material.

Gene and cDNA sequences for bovine BTN (**Genbank/EMBL accession numbers M35551; Z93323**) and BTN from some other species are available. At least ten related BTN genes have been identified in several species that have limited to broad expression profiles in tissues other than the mammary gland (e.g., Ye *et al*., 2000; Rhodes *et al*., 2001). Following the convention of the HUGO Gene Nomenclature Committee, the mammary-specific gene should be denoted *BTN1A1*, to distinguish it from related family members. BTN has an externally oriented N-terminus and a single trans-membrane anchor located close to the middle of the sequence (type 1 topology) (Jack and Mather, 1990; Banghart *et al*., 1998) (Figure 4.9). BTN and related family members belong to the immunoglobulin (Ig) superfamily of adhesive and receptor proteins. BTN has two Ig-like folds in the N-terminal half of the molecule. In the C-terminal part, there is a B30.2 domain, predicted to comprise two Ig folds (Sato *et al*., 1995), which may function as a protein-binding domain (Schweiger *et al*., 1999; Mather, 2000; Niikura *et al*., 2003; Zhai *et al*., 2004). As discussed in the section on secretion of milk fat globules, BTN associates with XDH, and probably with other proteins, to form a large complex.

It was long speculated that BTN plays some role in milk fat secretion (Mather and Jack, 1993) and now there is direct evidence for this. Ogg *et al*. (2004) prepared two lines of mice in which either variable amounts of a truncated form of BTN were expressed, or BTN expression was undetectable. Females of both lines were fertile and entered into lactation but had defects in fat globule secretion. In these mice, just like in ($Xdh^{+/-}$) mice, secretory cells became engorged with lipids. Lipid droplets were secreted with disrupted membranes and aggregated into large droplets.

ADPH was not identified as a major MFGM protein until recently because it co-migrates with the glycoprotein known as PAS 6/7. ADPH was known previously as adipocyte differentiation-related protein (ADRP) because it is expressed early during adipocyte differentiation and was believed to be expressed only in adipocytes (Jiang and Serrero, 1992). Since then, ADPH has been detected in a large number of tissues and cell types, where it invariably is associated with intracellular lipid droplets (Heid *et al*., 1996; 1998; Brasaemle *et al*., 1997).

ADPH migrates as an estimated 52 to 54 kDa polypeptide on SDS-PAGE (Figure 4.8). It remains largely associated with the MFGM, even

though it is not a trans-membrane protein, when globules are destabilized and it is recovered mostly, if not entirely, in the MFGM-associated coat material when membrane structure is destroyed (Heid *et al*., 1996). ADPH appears to be intermediate in abundance between BTN and XDH in bovine MFGM. This protein probably is not glycosylated (Heid *et al*., 1996).

The sequence of bovine ADPH was determined from its cDNA (**Genbank accession number AJ011680**); the bovine protein is 87 and 80% identical to human and mouse ADPH, respectively (Nielsen *et al*., 1999). Surprisingly, ADPH does not have an extensive contiguous sequence of hydrophobic amino acids. About half of the 50 N-terminal residues are nonpolar. McManaman *et al*. (2003) found that the lipid-targeting domain of ADPH is in a presumptive α-helical region between residues 189 and 205 of the mouse protein (Figure 4.9).

PAS 6/7, the bovine homolog of mouse MFG-E8, has been known by many names (Mather, 2000). This protein migrates as a broad doublet with an estimated molecular mass range of 43 to 58 kDa on SDS-PAGE (Figure 4.8). PAS 6/7 stains both with Coomassie blue and with the PAS reagent. PAS 6/7 is an extrinsic protein of the MFGM and can be largely displaced from the membrane by extraction with buffers containing a high concentration of salt (Mather, 2000). Upon destabilization of fat globules, a high proportion is recovered in the MFGM supernatant fraction.

The complete amino acid sequence derived from cDNA is available for bovine PAS 6/7 (**Genbank accession number X91895**) as well as the homologous protein from several other species (Mather, 2000). PAS 6/7 is the product of a single gene but gives rise to a doublet on SDS-PAGE because of variable glycosylation of an identical polypeptide chain. The protein contains both N-linked and O-linked glycan chains. In the N-terminal region of the protein, there are two epidermal growth factor-like domains, one of which contains the Arg-Gly-Asp adhesive sequence that binds to integrin receptors. PAS 6/7 also contains domains similar to the C1 and C2 domains of blood-clotting factors V and VIII (Figure 4.9). The C2 domain contains putative phospholipid-binding motifs and topological analysis suggests that PAS 6/7 is bound to anionic phospholipids of the MFGM (Mather, 2000). Sequence analysis of the cDNA of the rat homolog of PAS 6/7 provided evidence that this protein may be an acetyltransferase that functions in O-acetylation of ganglioside sialic acids (Ogura *et al*., 1996), but whether the MFGM protein has such enzymic activity remains to be determined.

PAS 6/7 has no known role in milk-fat globule secretion. The human homologue, lactadherin, binds to rotaviruses and may protect the gastrointestinal tract of the suckling young from infection (Peterson *et al*., 2001).

The mouse homologue, MFG-E8, plays a direct role in apoptosis as $MFG-E8^{-/-}$ mice are defective in the phagocytosis of apoptotic cells by macrophages (Hanayama *et al.*, 2004). MFG-E8 is secreted by activated macrophages and binds to phosphatidylserine expressed on the outer surface of apoptotic cells, thus stimulating their subsequent destruction by phagocytosis (Hanayama *et al.*, 2002).

FABP can be identified during SDS-PAGE of the MFGM as a protein that migrates with an estimated molecular weight of 13 kDa (Figure 4.8). Gels containing more than 10% polyacrylamide, or gradient gels, are required to resolve this protein from material that migrates at or near the dye front in gels of lower polyacrylamide concentration (Mather, 2000). This protein stains with Coomassie blue but not with the PAS reagent and, upon destabilization of fat globules, is recovered largely in the MFGM supernatant fraction. FABP was identified originally as a potent inhibitor of the growth of mammary carcinoma cells and was called "mammary-derived growth inhibitor" (Mather, 2000).

There are many tissue-specific or cell-specific forms of FABP; the MFGM form is most likely to be heart FABP, the sequence of which has been determined (**Swiss Protein accession number P10790**). However, it is possible that other forms of FABP also are associated with the MFGM (Mather, 2000). As yet, no functional role has been found for FABP of MFGM.

In addition to the major MFGM proteins, numerous other, quantitatively minor, polypeptides are detectable, especially when SDS gels are stained with silver (Figure 4.8). Two-dimensional separations reveal a complex pattern of minor constituent proteins (see, for example, Mather, 2000). Many of these constituents have yet to be identified in bovine MFGM (however, see Quaranta *et al.*, 2001, Cavaletto *et al.*, 2002 and Wu *et al.*, 2000 for proteomic studies of human and mouse MFGM). Some of these unidentified proteins are likely to be enzymes, as a large number of enzymic activities have been detected in MFGM (Table 4.3). In addition, one would expect that at least some of the proteins associated with the surface of lipid droplets within the cell are present in secreted milk-fat globules. However, the only such protein specificially identified is ADPH. Monomeric GTP-binding proteins associated with the MFGM have been identified by ligand binding but which of the minor silver-stained constituents migrating in the 19 to 25 kDa region correspond to these G-proteins remains to be determined. Recently, some preliminary evidence that a 23 kDa membrane constituent may be the C-terminal MUC1 heterodimeric partner and that a 43 kDa constituent may be actin has been obtained using antibodies for detection; the identity of actin was confirmed by partial sequence analysis (Heid and Keenan, 2005).

4.9. Molecular Organization of the MFGM

The organization of material on the fat globule surface has been the subject of much speculation and experimentation over the past about 90 years. Models of the nature and organization of the fat globule surface material have progressed from it being an adsorbed layer of constituents derived from milk serum to the current view that the MFGM is a true biological membrane. Five groups made seminal contributions leading to our current view of MFGM structure. Bargmann and Knoop (1959) were the first to provide electron micrographs showing that fat globules are secreted from cells by envelopment in apical plasma membrane. The biochemical studies of Morton and colleagues (Morton, 1954; Bailie and Morton, 1958a,b) and of Brunner and colleagues (Dowben *et al*., 1967) provided evidence that the MFGM contains a number of enzymes typically found in cellular membranes. Patton and Fowkes (1967) showed that the phospholipids of MFGM are like those of cellular membranes and provided a biophysical rationale for the envelopment of lipid droplets in plasma membrane. Patton and Trams (1971) and Mather and Keenan (1975) showed that certain MFGM proteins are disposed assymetrically with respect to the plane of the lipid bilayer of the membrane.

Our current view of the MFGM is that it is a true bilayer membrane with a dense protein coat 10 to 50 nm thick oriented on the cytoplasmic membrane face (the face contacting the core lipids of the globule) and an innermost layer derived from material that coated the lipid droplets before secretion. The bilayer membrane of the MFGM almost certainly is derived from specialized regions of apical plasma membrane. The dense protein coat is most probably the complex formed from interaction of cytosolic XDH with the cytoplasmic tails of BTN and ADPH and perhaps other proteins of the intracellular lipid droplet surface (Figure 4.9).

Early morphological and biochemical studies showing that there is a distinct asymmetric orientation of the glycans, enzyme-active sites, and proteins of the MFGM have been in part confirmed by molecular biological approaches (Figure 4.9). With respect to the major proteins, it has been established that PAS 6/7 is an externally disposed extrinsic MFGM constituent and that XDH is an internally disposed constituent of the MFGM coat that interacts with the membrane by association with BTN (for review, see Mather and Keenan, 1998). MUC1, MUC15 and BTN are trans-membrane proteins with single membrane-spanning regions and with externally-oriented N-termini. CD36 also is a trans-membrane protein but has short, internally-disposed N-terminal and C-terminal segments and a large, externally-disposed loop. Apparently, ADPH does not occur in plasma membrane

(Heid *et al.*, 1996), so it is likely that this protein originates from the surface of intracellular lipid droplets and interacts with BTN, XDH, or perhaps other proteins or protein complexes on the inner face of the MFGM. Phospholipids and glycosphingolipids are known to be asymmetrically organized in cellular membranes but we have no specific information as to how these constituents are oriented in the MFGM.

4.10. Perspectives

Much of the progress made in this research area during the past 10 years has been in elucidating the sequence and orientation of MFGM proteins and establishing that XDH and BTN are necessary for the normal secretion of milk-fat globules. Data suggestive of factors that regulate the actual milk-fat globule secretory process have also become available. In contrast, there has been little advance in our knowledge of how intracellular precursors of milk lipid globules form and grow prior to secretion. We now have a knowledge base sufficient to allow drawing of diagrams showing the origin, growth, transit, and secretion of milk fat globules (Figure 4.5) but there is a severe lack of understanding of the molecular mechanisms involved in these processes. This research area is ripe for the application of molecular cell biological approaches to answer the many remaining questions.

One major obstacle to molecular studies in the area of milk-fat globule formation and secretion is the lack of a cell line that secretes, or can be induced to secrete, milk fat globules, although some progress in this area has been reported (Rohlfs *et al.*, 1993). Development of such a cell line would be a major advance, in and of itself, and would be an invaluable aid in fostering further research in this area.

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