Antibodies to the Major Insoluble Milk Fat Globule Membrane-associated Protein: Specific Location in Apical Regions of Lactating Epithelial Cells

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ABSTRACT

Milk lipid globules of various species are surrounded by a membrane structure that is separated from the triglyceride core of the globule by a densely staining fuzzy coat layer of 10- to 50-nm thickness. This internal coat structure remains attached to the membrane during isolation and extraction with low- and high-salt buffers, is insoluble in nondenaturing detergents, and is enriched in an acidic glycoprotein (butyrophilin) with an apparent M_r of 67,000. Guinea pig antibodies against this protein, which show cross-reaction with the corresponding protein in some (goat) but not other (human, rat) species, have been used for localization of butyrophilin on frozen sections of various tissues from cow by immunofluorescence and electron microscopy. Significant reaction is found only in milk-secreting epithelial cells and not in other cell types of mammary gland and various epithelial tissues. In milk-secreting cells, the staining is restricted to the apical cell surface, including budding milk lipid globules, and to the periphery of the milk lipid globules contained in the alveolar lumina. These findings indicate that butyrophilin, which is constitutively secreted by surface budding in coordination with milk lipid production, is located at the apical surface and is not detected at basolateral surfaces, in endoplasmic reticulum, and in the Golgi apparatus. This protein structure represents an example of a cell type-specific cytoskeletal component in a cell apex. It is suggested that this antigen provides a specific marker for the apical surface of milk-secreting cells and that butyrophilin is involved in the vectorial discharge of milk lipid globules.

Lactating mammary gland has received increasing attention as a model for studies of secretory mechanisms and membrane dynamics. Two major secretory mechanisms operate in milk-secreting mammary epithelial cells. Lactose and milk proteins such as α-lactalbumin and caseins appear to be packaged together in Golgi apparatus–derived secretory vesicles and exit from the cell by the process of exocytotic fusion of secretory vesicle membrane with the apical plasma membrane (see references 13, 28, and 41, and references cited therein). In contrast, morphological and biochemical evidence indicates that milk lipid globules are enveloped in apical plasma membrane during their extrusion from the cells (2; for reviews, see references 26, and 37). Thus, formation of milk fat globule membrane (MFGM) provides an opportunity to study selective budding of regions of apical surface membrane and selective accumulation or exclusion of membrane proteins from regions of membrane that participate in envelopment of a cytoplasmic component, here the lipid droplet (26, 37, 38, 49).

On electrophoretic separation, MFGM from different species have been found to contain a limited number of size classes of major polypeptides (20, 31, 32). Two of these polypeptides (band 3, apparent M_r 155,000, containing xanthine oxidase [cf. reference 33]; and band 12, apparent M_r 67,000) have been found to be enriched in the salt- and water-insoluble material
of the dense coat tightly associated with the inner face of MFGM (20). This coat material can be obtained as an insoluble fraction resistant to extraction of MFGM with high-salt buffers, various detergents, or chaotropic agents (20). The predominant polypeptide of this coat material (band 12, Mr 67,000) has been partially characterized and has been found to be an acidic glycoprotein that tenaciously retains small amounts of mem-

**Electrophoresis and Immunological Detection of Proteins**

Polyacrylamide gel electrophoresis in the presence of SDS was as described (16, 20). Two-dimensional gel electrophoresis was performed essentially according to O'Farrell (35). Samples were solubilized in lysis buffer (35) directly or after special precautions such as short incubations in PBS solutions or in PBS containing 100 mM MgCl₂ (14, 30) were taken in order to minimize artificial losses during incubation. Fluorescein isothiocyanate (FITC)-conjugated goat antibodies against rabbit IgG and FITC-conjugated rabbit antibodies against guinea pig IgG or murine IgG were used as second antibodies (Miles-Ueda, Rehovot, Israel).

**Immunofluorescence Microscopy**

Procedures for indirect immunofluorescence microscopy on cryostat sections were as described (15). Sections were air-dried and/or fixed in -20°C cold acetone or, alternatively, fixed with 2% formaldehyde freshly prepared from paraformaldehyde in phosphate-buffered saline (PBS). In some experiments, special precautions such as short incubations in PBS solutions or in PBS containing 100 mM MgCl₂ (14, 30) were taken in order to minimize artificial losses during incubation. Fluorescein isothiocyanate (FITC)-conjugated goat antibodies against rabbit IgG and FITC-conjugated rabbit antibodies against guinea pig IgG or murine IgG were used as second antibodies (Miles-Ueda, Rehovot, Israel).

Mammary tissue from lactating Holstein cows, obtained from a local slaughterhouse, was immediately frozen in isopentane cooled with liquid nitrogen and stored at -70°C until processing for immunofluorescence microscopy (cf. reference 14). Liver, muzzle, and small intestine tissues were also obtained and rapidly frozen. Calf thymus was similarly processed. MFGM were prepared as in previous studies (20, 25, 32), and coat fractions were obtained from MFGM by extraction with 1% Triton X-100 in 10 mM Tris-HCl, pH 7.4 (20). For comparison, MFGM fractions from other species (human, goat, rat) were similarly prepared (20).

**Materials and Methods**

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**Antibodies**

Band 12 protein was obtained by electrophoresis in slab gels containing SDS and was recovered from gel slices as described (3, 14, 18, 20). Antibodies directed against this protein were elicited in guinea pigs following an immunization schedule essentially as described (19, 20). The IgG fraction was isolated from this serum by ammonium sulfate precipitation and DEAE cellulose chromatography (14). In addition, rabbit and mouse antibodies against band 12 protein described previously (19, 20) were used. Specific rabbit antibodies against cytochrome b₅ against actin, and against the total prekeratin fraction of desmosome-attached tonofilaments from bovine muzzle were as described (8, 14). For controls, preimmune sera and IgG fractions were used.

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**Electron Microscopy**

Isolated MFGM and coat fractions made therefrom were fixed and processed for electron microscope examination of ultrathin sections as in previous studies (13, 20, 25).

For immunoperoxidase localization studies, sections of frozen tissues prepared as described above were air-dried and briefly (5 min) treated with cold (-20°C) acetone. The dried sections were incubated with antibodies (~50 μg/ml purified IgG) for 45 min at room temperature. After three washes with PBS, each for 5 min, the sections were allowed to react with peroxidase-conjugated rabbit immu

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treatment with low concentrations of SDS as described by Kelly and Cotman (29). Alternatively, samples were dissolved by boiling for 7 min in 10 mM sodium-potassium-phosphate buffer (pH 7.5) containing 5% SDS and 10% 2-mercaptoethanol, cooled to room temperature, and cleared by centrifugation at 18,000 g. The supernatant was precipitated with 9 vol of acetone at -20°C, and the precipitate was washed, by resuspension and centrifugation first with -20°C cold acetone-water (9:1, vol/vol), and then with -20°C cold 96% acetone. The final pellets were dried under N₂ and kept dry until solution in lysis buffer (35).

Gels were stained with Coomassie Blue or, for detection of glycoproteins, with the periodic acid–Schiff (PAS) reaction (cf. reference 20).

Immunoreplicas using agarose gel overlays were made as described (14, 16, 19). Alternatively, proteins separated by gel electrophoresis were transferred to nitrocellulose paper sheets by blotting for 24 h at room temperature essentially according to Towbin et al. (43). The sheets were soaked in 1% bovine serum albumin (BSA) in PBS for 12 h at room temperature, rinsed three times with PBS, and incubated for 1 h at room temperature with the specific solution of guinea pig antibodies diluted 1:100 with PBS containing 2% BSA. Thereafter, the sheets were washed five times with PBS, followed by one wash in 10 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl and one wash in 10 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl. They were then incubated for 2 h at room temperature with 125I-labeled protein A in PBS containing 2% BSA (total radioactivity per sheet: 0.5-1.0 TCI). To remove unbound protein A, the sheets were washed first with 125I-labeled protein A in PBS containing 2% BSA (total radioactivity per sheet: 0.5-1.0 TCI). To remove unbound protein A, the sheets were washed first five times with PBS, followed by one wash in 10 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl, and one wash in 10 mM Tris-buffer containing 0.15 M NaCl. They were then incubated for 2 h at room temperature with 125I-labeled protein A in PBS containing 2% BSA (total radioactivity per sheet: 0.5-1.0 TCI). To remove unbound protein A, the sheets were washed first five times with 0.5% Triton X-100 in 10 mM Tris-buffer (pH 7.4) for 1 h at room temperature, then in 10 mM Tris-buffer containing 0.5 M NaCl, and finally in 10 mM Tris-buffer containing 0.15 M NaCl. The washed sheets were dried between filter paper at 80°C for 15 min and were exposed to Kodak X-Omat R film at -70°C.

RESULTS

Gel Electrophoresis of MFGM and Characterization of Antibodies

Intact milk lipid globules are surrounded by a membrane that has a typical unit membrane appearance and is derived from the apical plasma membrane. Usually, a thick coat (10-50 nm) of densely staining material is observed between the inner face of the MFGM and the outer shell of the lipid droplet (13, 20, 37, 45). This internal coat material is maintained on MFGM during isolation and extensive washing (Fig. 1). Two major polypeptides (band 3, Mr 155,000, xanthine oxidase; and band 12, Mr 67,000, butyrophilin; Fig. 2) are selectively enriched in coat preparations obtained by treatment of MFGM with detergents (20). These two polypeptide bands are also prevalent in MFGM from other species (illustrated for goat in Fig. 2 and human milk in Fig. 3; see also references 20 and 34). On two-dimensional gel electrophoresis of total MFGM, of residual MFGM coat material extracted with high-salt buffer and Triton X-100, or of purified band 12 protein obtained by excision from gels, elution and acetone treatment (see Materials and Methods), butyrophilin appears as an acidic protein (cf. reference 31) and, in cow's milk, reveals at least four distinct isoelectric variants focusing at approximate pH values of 5.30, 5.27 (major component), 5.24, and 5.22 (Fig. 4a and b). No other protein of Mr 67,000 is seen on two-dimensional gel electrophoresis of whole MFGM or band 12 protein preparations excised from SDS polyacrylamide gels (Fig. 4a). When such gels are stained using the PAS reaction, the whole series of isoelectric variants is positively stained (Fig. 4c), confirming and extending previous findings from our laboratories (20) and from Mather (31) that butyrophilin is glycosylated. By contrast, xanthine oxidase (Fig. 4a), contained in "band 3 protein" of MFGM, is not significantly stained with the PAS reaction (20, 31), even after loading (not shown here; this protein has several variants with apparent isoelectric pH values in the range from 7.3 to 7.7; cf. reference 31).

Guinea pig antibodies to bovine band 12 protein eluted from SDS polyacrylamide gels were found to be positive by immunodiffusion analysis using the procedure described by Yen et al. (47) for solubilization of antigen (data not shown). Antisera and IgG fractions were also characterized by the immunoreplica and "immunoblot" methods. The antibodies reacted strongly and specifically with the Mr 67,000 polypeptide present...
in bovine MFGM (Figs. 3 and 4d) and, after two-dimensional gel electrophoresis, it was shown that the antibodies reacted with the whole range of isoelectric variants (Fig. 4d). The corresponding butyrophilin in goat MFGM also reacted with the antibodies but the $M_t$, 67,000 polypeptide of human MFGM did not react (Fig. 3). Likewise, no cross-reaction was seen between the $M_t$, 67,000 polypeptides of bovine and rat MFGM (not shown; see also references 20 and 34).

**Light Microscopy**

When frozen sections of lactating bovine mammary gland were examined by immunofluorescence microscopy using antibodies to butyrophilin, strong staining was observed over regions of epithelial cells that bordered alveolar lumina (Fig. 5). In addition, structures within lumina were also stained, which allowed positive identification of milk lipid globules extruded from cells (Figs. 5a and 6a and b). The inset in Fig. 5a shows a putative lipid globule fixed during extrusion into the lumen to be stained entirely with antibodies to butyrophilin. Positive staining was also seen in the most distal parts of budding milk lipid globules (Fig. 6a). In addition, "punctate" fluorescence in small particles was observed within the alveolar lumina and might be attributed to MFGM fragments sloughed from milk fat globules during "aging" of secreted globules or during fixation (for electron microscopy indicating delamination of MFGM material, see, e.g., reference 45). Punctate discontinuities of fluorescence were also seen around some of the milk fat globules, nascent or postsecretory, and might reflect either sloughing of parts of the MFGM material (cf. reference 43) or natural small discontinuities of the internal coat layer as described by electron microscopy (e.g., reference 20; see also Fig. 9c). Intracellular lipid droplets, which lack a continual membrane envelope (e.g., references 2, 13, 37, and 45), did not appear to be surrounded by the antigen (Fig. 5b and c). Myoepithelial cells and cells within the lamina propria were not stained with these antibodies. Although apical regions of epithelial cells were intensely stained (Figs. 5 and 6a and b), we never observed staining over basal and lateral regions of epithelial cells, suggesting that butyrophilin is selectively located in apical regions of epithelial cells and on luminal lipid globules. Similar immunofluorescence staining was found after the various modifications of incubation with antibodies mentioned in Materials and Methods, indicating that little, if any, of this antigen was lost or inactivated during the preparation. Rabbit and mouse antibodies against bovine butyrophilin gave practically identical results, showing strong staining of the apical rim of milk-secreting cells (not shown here; an example for murine antibodies has been presented in reference 19). Control experiments in which preimmune serum or IgG were used as first antibodies showed no significant fluorescence (Fig. 6c).

Staining patterns observed with antibodies to butyrophilin were different from those observed with antibodies to other apically enriched proteins such as tonofilamentous cytokeratin and actin (Fig. 6d-f). Antibodies against prekeratin and actin also did not stain structures within alveolar lumina (Fig. 6d and f). Prekeratin antibodies gave intense fluorescence in myoepithelial cells (Fig. 6d; see also references 14 and 42) and in lactating epithelial cells, where they appeared to be especially enriched at small foci of fluorescence coincident with desmosomal contacts (arrows in Fig. 6d). These foci of fluorescence most probably represent the short tufts of desmosome-attached tonofilaments that, in the cow but not in rodents, are seen in these regions (for electron microscopy evidence, see reference 48). Similarly, antibodies to actin intensely stained the myoepithelial cells (Fig. 6e and f) as described previously (14) but also, in agreement with our earlier study (14), apical regions of epithelial cells. It is in this region that actin-containing micro-

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filaments are most commonly observed (13). Staining with antibodies to actin was not seen over lipid globules or over luminal areas (13), in agreement with the demonstrated absence of considerable amounts of actin in MFGM (27). However, regions just under budding lipid globules bound antibodies to actin (Fig. 6f, inset), in agreement with our electron microscope observation that microfilament bundles can occur in basal ("stalk") regions of budding lipid globules in arrays reminiscent of microfilament organization in cleavage furrows. By contrast, antibodies to cytochrome $b_6$ showed strong staining over the whole cytoplasm (Fig. 7), as previously shown for rat mammary gland (8).

Several other bovine tissues, such as liver, muzzle epidermis, small intestine, and thymus, that were examined by immuno-
fluorescence microscopy after application of antibodies to butyrophilin were observed to be negative. The absence of positive localization in apical regions of other polarized epithelial cells such as small intestine (Fig. 8) deserves comment. Distribution of actin microfilaments and various associated proteins present in microvilli and terminal webs of intestinal epithelium has been intensively studied (e.g., references 5, 6, 9, 21). In particular, a major apical cytoskeleton protein of Mr 68,000, for which the name fimbrin has been proposed (7), has been shown to be localized in brush borders of small intestine. Our observation that butyrophilin antibodies do not significantly stain intestinal epithelial cells distinguishes this protein from fimbrin and further illustrates the specificity of butyrophilin for the lactating mammary epithelial cell. Both ultrastructure and immunolocalization also distinguish butyrophilin of mammary gland from other insoluble apical proteins such as the desmosome-tonofilament meshwork of the "apical skeletal disk" described in intestinal epithelium (11).

Electron Microscopy

The reaction of butyrophilin antibodies with components of lactating mammary gland cells of the cow's udder was also examined at the electron microscopic level, employing the immunoperoxidase method on frozen sections that had been dried and processed in a manner similar to that for immunofluorescence microscopy (see Materials and Methods). Light microscope controls demonstrated that the immunoperoxidase procedure gave the same results as the immunofluorescence technique, showing enrichment, if not exclusive localization, of the antigen in apical regions of milk-secreting epithelial cells (Fig. 9a). As indicated by the inset to Fig. 9a, DAB reaction within this zone can be found on the apical side of budding milk fat globules. This distribution of staining was also found on electron microscope examinations of such preparations, using ultrathin sections without any additional staining (Fig. 9b and c): strong staining was only seen on apical plasma membrane including surfaces of nascent milk fat globules (Fig. 9b). No significant staining was detected on basolateral plasma membrane regions, in all other intracellular membranes, or in nuclei (Fig. 9b). Negative reaction of butyrophilin antibodies was also observed in myoepithelial cells (Fig. 9b) and in all cell types present in the lamina propria and blood capillaries (not shown here). Higher magnification of the luminal surface region of budding milk fat globules clearly showed that the entire 10- to 50-nm-thick cytoplasmic coat layer underneath the surface membrane was positively stained by DAB reaction product, except for some occasional small discontinuities.
known to occur on milk fat globule surfaces from various species, human included (20).

DISCUSSION

We have identified, by immunofluorescence and electron microscopy, an antigen that is located in the apical region of milk-secreting epithelial cells and around the lipid globules found in alveolar lumina of mammary gland and is recognized by antibodies to butyrophilin from bovine MFGM. From our observations it is apparent that the constituent recognized by these antibodies does not occur, in amounts detectable by this technique, in any other cells present in mammary gland. Within the secretory epithelial cells this antigen is concentrated, if not exclusively located, in apical regions of the cell, prominently in a dense coat covering the cytoplasmic aspect of the plasma membrane. The visualization, by immunofluorescence microscopy, of such a structure of a thickness (10-50 nm) much below the resolution of the light microscope, as a relatively wide, intensely fluorescent rim is not surprising in view of the visualization of even thinner structures such as individual microtubules (see, e.g., reference 36) that have been converted to “self-emitting” fluorescent objects by binding of FITC-conjugated IgG. Because the butyrophilin antigen is also present around extracellular lipid globules, it is most probably a constituent of the apical plasma membrane and/or the dense cytoplasmic coat associated with it. Detectable amounts of the antigen are not present in basal or lateral regions of these cells or in the supranuclear cytoplasm where the Golgi apparatus is located and endoplasmic reticulum is particularly abundant (2, 4, 13, 23). However, the observation of an absence of reaction in intracellular membranes does not necessarily provide proof that the protein is completely absent from these membranes: it could be present in very small amounts, or the specific determinant(s) could be lacking or masked in a special mode. On the other hand, we have shown that endoplasmic reticulum membranes, for example, are accessible to immunoglobulins when mammary gland tissue is prepared as in this study, as shown by the strong staining with antibodies to cytochrome b₅ (Fig. 7; see also reference 8).

It has been suggested or implied by other workers that MFGM originates, at least to a considerable degree, directly from secretory vesicles or Golgi apparatus without first being integrated into apical plasma membranes (24, 39, 44, 46). Although our results do not exclude the possibility of some contribution from other cell membranes to the formation of MFGM, they appear to strongly favor the concept that apical plasma membrane is the major source of the membrane complex enveloping the milk lipid globules. Especially interesting in this context is our observation that butyrophilin is localized nearly over the entire apical surface and is not restricted to regions engaged in budding. This indicates that the structure contributing this insoluble apical protein is associated with the surface membrane and is not only formed as the result of a local interaction of the apical membrane with a milk lipid droplet.

That this antigen has not been observed in other tissues or cell types suggests that butyrophilin is a marker specific for the apical plasma membrane of lactating mammary epithelial cells. A wide tissue survey for this protein coupled with immunolocalization will be necessary to test this concept. The biological function of butyrophilin is not known. Nevertheless, it is tempting to speculate that this protein, which is so abundant in MFGM, is a plasma membrane-associated cytoskeletal protein functionally involved in the recognition, budding, and vectorial discharge of milk lipid globules at the cell apex.

As mentioned above, butyrophilin has some unusual properties: (a) It is enriched in “cytoskeletal” fractions obtained after extraction with high- and low-salt buffers and nonionating detergents (20). (b) Unlike other cytoskeletal proteins, however, it contains some carbohydrates, notably mannose, glucosamine, and galactose. Our data indicate that a considerable proportion, if not all, of this protein, or at least the antigenic determinant recognized, is contained in the dense coat covering the internal side of the MFGM. At present, however, we do not know how the carbohydrate residues of butyrophilin are oriented, relative to the plasma membrane surface. The molecular location of the carbohydrate residues and the antigenic determinants in relation to the MFGM coat also remains to be determined. The simplest interpretation, that butyrophilin might be a glycosylated protein associated with the cytoplasmic side of apical plasma membrane, is in obvious conflict with current concepts of biosynthesis and location of glycoproteins. However, there are several other possible ways by which this protein could be formed and organized: For example, butyrophilin could be an integral transmembrane protein that is glycosylated in the part of the molecule exposed on the external surface while the other, probably larger, part of the molecule is exposed on the cytoplasmic side and a constituent of the coat. On the other hand, the localization of butyrophilin in the cytoskeletal coat structure on the inner aspect of the apical plasma membrane could as well reflect an unusual rearrangement of membrane glycoprotein(s) in a special locally restricted structure, or the unusual glycosylation of a cytoskeletal protein in a special structural complex. Future experiments on the biosynthesis of butyrophilin are necessary to the understanding of the nature and origin of this remarkable protein.

FIGURE 8 Immunofluorescence microscopy of frozen sections through bovine small intestine as seen in oblique (a) and cross (b) sections of intestinal villi after reaction with antibodies to band 12 protein of bovine MFGM. Note absence of significant staining. Bars, 25 μm.
of sections through frozen and dried tissue of cow's udder processed using guinea pig antibodies (purified IgG) to butyrophilin from bovine milk for localization of butyrophilin by the immunoperoxidase method.

**FIGURE 9** Light microscopy (a) and electron microscopy (b and c) of sections through frozen and dried tissue of cow's udder processed for localization of butyrophilin by the immunoperoxidase method using guinea pig antibodies (purified IgG) to butyrophilin from bovine milk. Light microscopy (a, bright field) of the immunoperoxidase reaction gives the same result as described for immunofluorescence microscopy. J. Cell Biol. 74:204-225.

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