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

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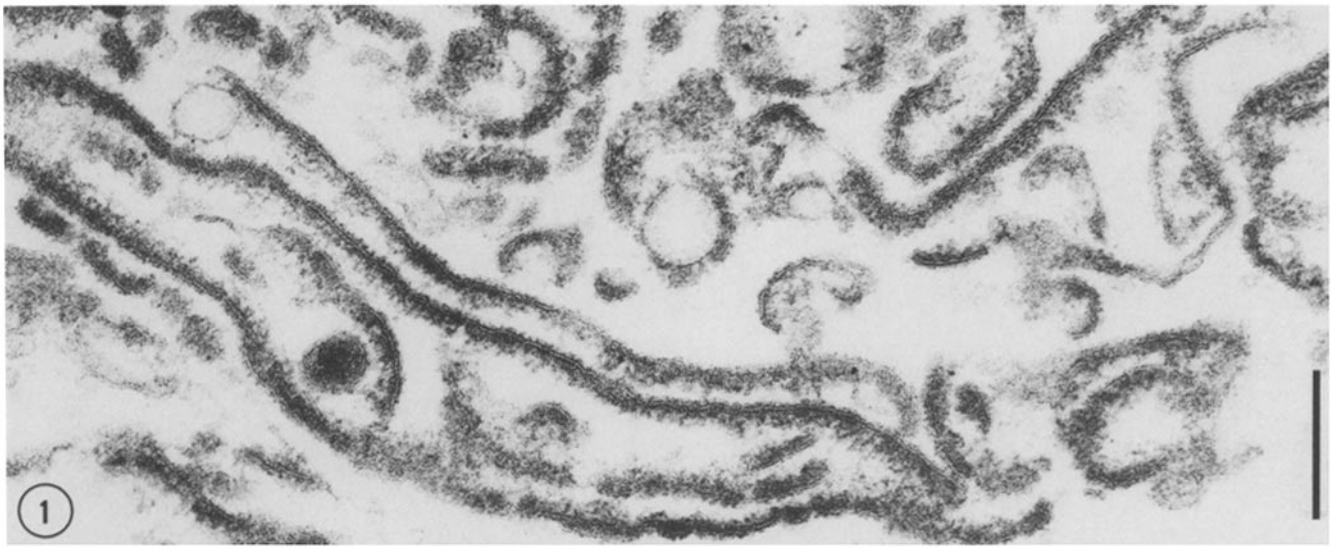


FIGURE 1 Electron micrograph of a section through a pellet of isolated and washed bovine milk fat globule membranes (MFGM), showing membrane sheets with typical "unit-membrane" appearance and densely stained fuzzy coat on one surface, i.e., the previously internal side. Bar, 0.2 μm. × 100,000.

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, M_r 67,000) has been partially characterized and has been found to be an acidic glycoprotein that tenaciously retains small amounts of membrane phospholipids (20, 31). This protein therefore appears to present the unusual example of a glycoprotein that is insoluble in detergents, is associated with a structure located on the cytoplasmic face of the surface membrane, and may function in budding of milk lipid globules. Because it is continually lost from the milk-secreting cell, it must be synthesized at relatively high rates, in coordination with the production of the milk lipids. In this communication we present evidence by immunolocalization that the band 12 protein, for which the name butyrophilin[1] is proposed, is a specific marker for the apical cell cortex of milk secreting epithelial cells.

MATERIALS AND METHODS

Materials

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).

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 (18, 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 b_5, 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.

[1] Meaning "associated with milk fat" as well as "having affinity to milk fat"; from the Greek words τό βούτυρον for butter, and φίλος, meaning: "liking something, being liked, belonging to, being related to."

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 $MgCl_2$ (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).

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 immunoglobulins against guinea pig IgG (Cappel Labs., Cochranville, Pa.) for another 45 min at room temperature. After three washes in PBS the samples were fixed for 10 min with either 2% formaldehyde (freshly made from paraformaldehyde) or 2.5% glutaraldehyde, both in PBS. Fixed samples were then washed with 50 mM Tris-HCl buffer (pH 7.6) and allowed to react for 10 min with a solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Serva, Heidelberg, Germany; 5 mg of DAB in 10 ml of 50 mM Tris-HCl, pH 7.6) to which 5 μl of 30% solution of H_2O_2 had been added immediately before use. After two washes with distilled water, the samples were treated wtih 1% aqueous osmium tetroxide for 30 min. After being washed with distilled water, the sections were dehydrated, embedded, and processed as described for flat-embedding of tissue culture cells grown on cover slips (12). Ultrathin sections obtained from such preparations were either "double-stained" with uranyl acetate and lead citrate or were not stained at all. For evaluation of the significance of immunoperoxidase staining only unstained ultrathin sections were used.

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

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 supernate 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 N_2 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).

Immunoreplicae 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 ^{125}I-labeled protein A in PBS containing 2% BSA (total radioactivity per sheet: 0.5–1.0 μCi). 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, M_r 155,000, xanthine oxidase; and band 12, M_r 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. 4 *a* and *b*). No other protein of M_r 67,000 is seen on two-dimensional gel electrophoresis of whole MFGM or band 12 protein preparations excised from SDS polyacrylamide gels (Fig. 4 *a*). When such gels are stained using the PAS reaction, the whole series of isoelectric variants is positively stained (Fig. 4 *c*), confirming and extending previous findings from our laboratories (20) and from Mather (31) that butyrophilin is glycosylated. By contrast, xanthine oxidase (Fig. 4 *a*), contained in "band 3 protein" of MFGM, is not significantly stained with the PAS reaction (20, 31), even after overloading (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 M_r 67,000 polypeptide present

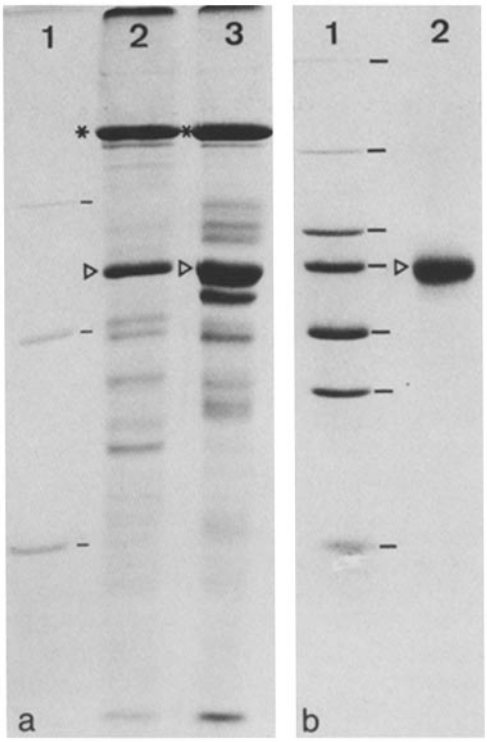


FIGURE 2 SDS polyacrylamide gel (7.5%) electrophoresis of whole MFGM proteins (*a*) from goat (slot *2*) and cow (slot *3*), in comparison with reference proteins (bars in slot *1*, from top to bottom: phosphorylase *a*, glutamate dehydrogenase, and chymotrypsinogen). Note the predominance of proteins of band 3 (asterisk) containing xanthine oxidase and band 12 (triangle) containing butyrophilin. The purified band 12 protein used as antigen is shown in slot *2* of *b*, in comparison with reference proteins (slot *1* in *b*; bars denote, from top to bottom: myosin, β-galactosidase, transferrin, BSA, glutamate dehydrogenase, actin, and chymotrypsinogen).

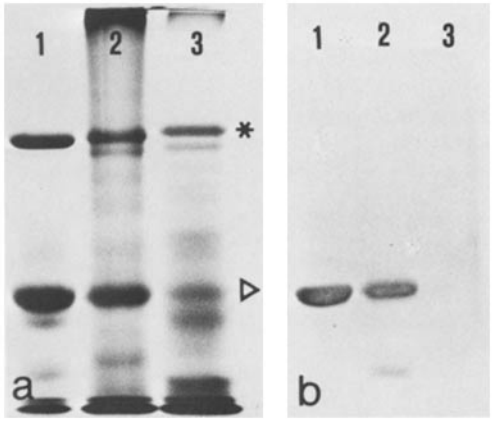


FIGURE 3 SDS polyacrylamide gel electrophoresis (*a*) of total MFGM proteins of bovine (slot *1*), caprine (slot *2*), and human (slot *3*) milk (asterisk denotes position of band 3 protein; triangle denotes position of band 12 protein), and the corresponding agarose overlay immunoreplica (*b*), showing that specific immunoprecipitates are obtained after reaction of guinea pig antibodies to butyrophilin with band 12 protein from bovine (slot *1*) and caprine (slot *2*) but not human (slot *3*) MFGM.

in bovine MFGM (Figs. 3 and 4*d*) and, after two-dimensional gel electrophoresis, it was shown that the antibodies reacted with the whole range of isoelectric variants (Fig. 4*d*). The corresponding butyrophilin in goat MFGM also reacted with the antibodies but the M_r 67,000 polypeptide of human MFGM did not react (Fig. 3). Likewise, no cross-reaction was seen between the M_r 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. 5*a* and 6*a* and *b*). The *inset* in Fig. 5*a* 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. 6*a*). 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. 9*c*). 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. 5*b* 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 6*a* 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. 6*c*).

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. 6*d–f*). Antibodies against prekeratin and actin also did not stain structures within alveolar lumina (Fig. 6*d* and *f*). Prekeratin antibodies gave intense fluorescence in myoepithelial cells (Fig. 6*d*; 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. 6*d*). 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 microscope evidence, see reference 48). Similarly, antibodies to actin intensely stained the myoepithelial cells (Fig. 6*e* 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-

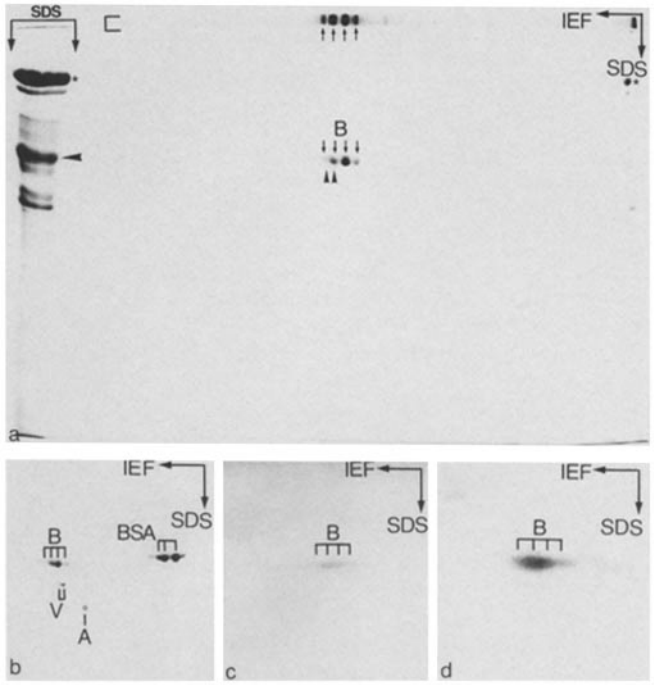


FIGURE 4 Two-dimensional gel electrophoretic separation of polypeptides of total washed bovine MFGM showing that butyrophilin (*B*), which occurs in four major isoelectric variants (short arrows in *a*, brackets designated *B* in *b–d*), is the by far predominant acidic protein present in this structure. Arrows in the upper right indicate direction of isoelectric focusing (IEF) and electrophoresis in the presence of SDS in the second dimension. A corresponding parallel IEF gel (first dimension) is shown separately at the top margin (bracket denotes the diameter of the gel cylinder); a one-dimensional separation of total MFGM polypeptides (same sample) has been run, together with the second dimension of the two-dimensional gel electrophoresis, on the left margin (denoted in *a* by the bracket with arrows and the heading "SDS"). Asterisks denote the position of "band 3 protein" containing xanthine oxidase activity. The large arrowhead on the left gel track denotes the position of band 12 protein containing butyrophilin. The small arrowheads denote isoelectric variants of "band 13 protein" that have been immunologically identified as breakdown products of butyrophilin, probably by proteolysis (not shown here; see also reference 20). The appearance of butyrophilin (*B*) on two-dimensional gel electrophoresis, relative to co-electrophoresed acidic reference proteins, is shown in *b*. In addition to co-electrophoresed bovine serum albumin (*BSA*) (apparent isoelectric pH range of the three major isoelectric variants: 6.2–6.4; for similar values, see references 1 and 10) and murine vimentin (*V*) (isoelectric pH of major unphosphorylated variant in several mammalian species, cow included: 5.30; cf. references 17, 22, and 40), the relative position of α-actin (*A*) from rabbit skeletal muscle as seen in a parallel electrophoresis is indicated. *a* and *b* have been stained with Coomassie Blue. For comparison, the PAS reaction of a gel electrophoretic separation similar to that shown in *a* is presented in *c*, illustrating the carbohydrate content of the whole range of isoelectric variants of butyrophilin (*B*). Correspondingly, the reaction of the series of isoelectric variants of butyrophilin, seen after two-dimensional gel electrophoresis (*a–c*), with the guinea pig antibodies used in this study is demonstrated in the radioautograph of a nitrocellulose blot-immunoreaction using ^{125}I-labeled protein A for detection of immunoglobulins (*d*).

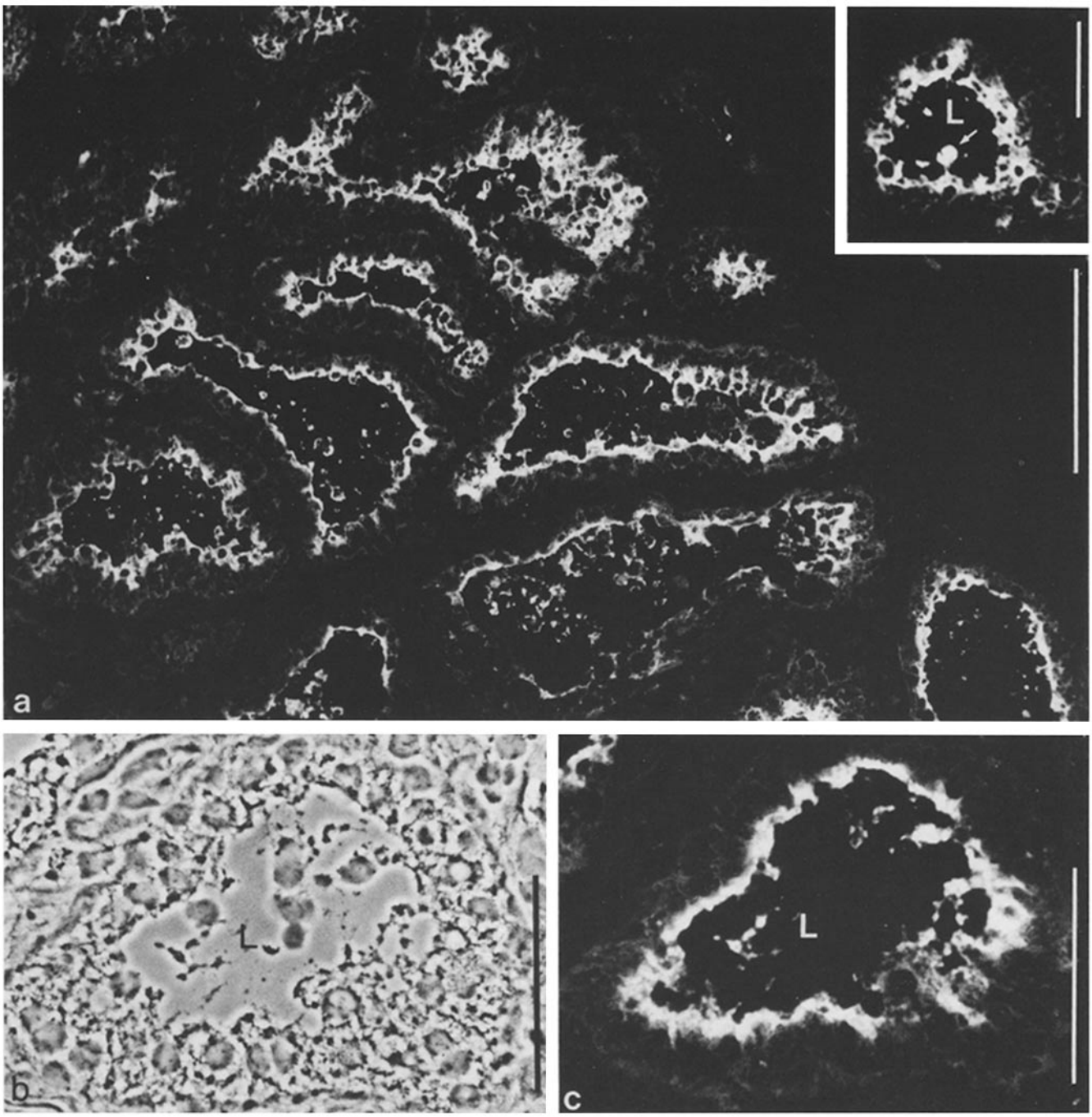
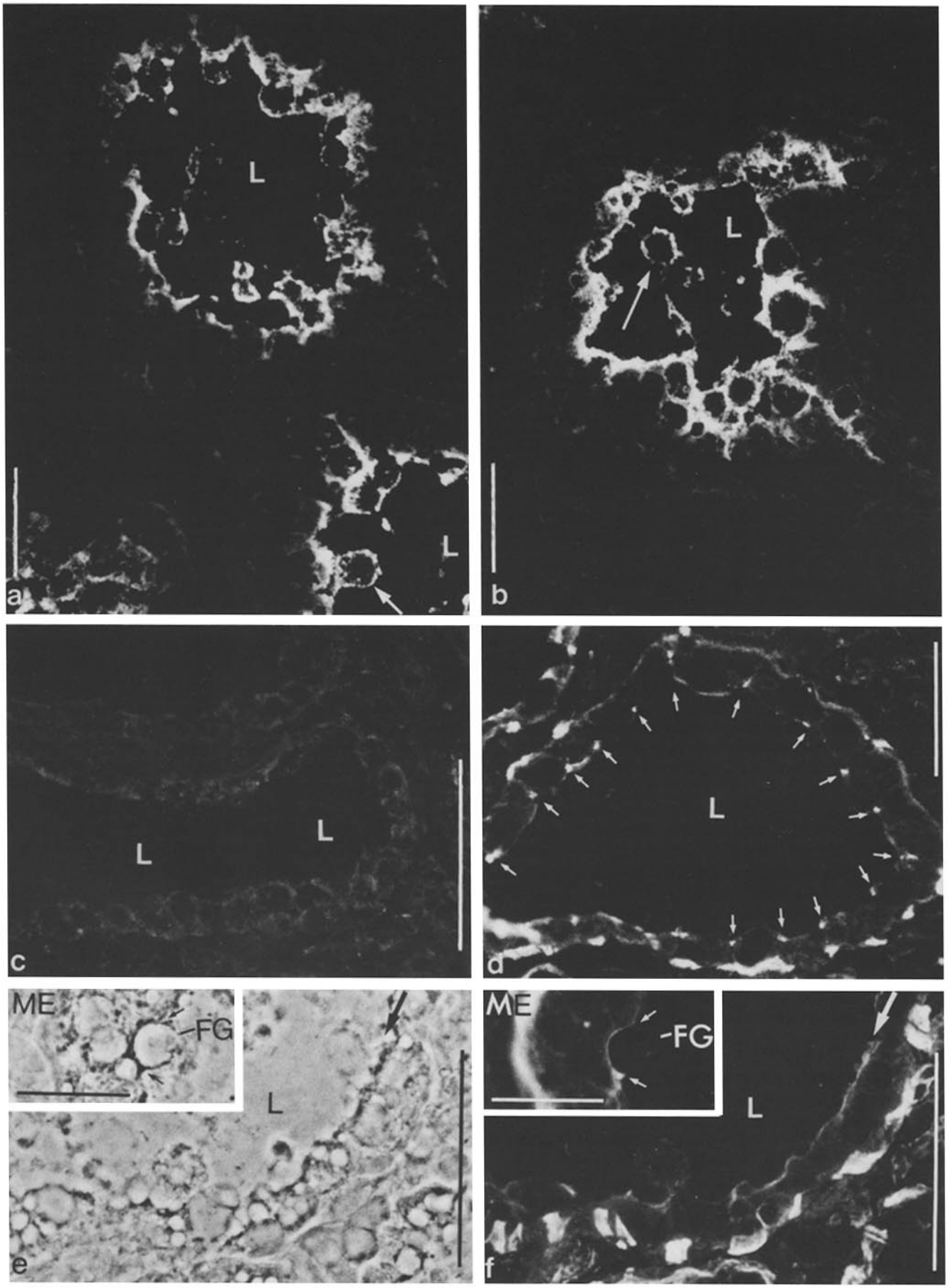


FIGURE 5 Immunofluorescence microscopy of frozen sections through lactating cow's udder after staining with antibodies to butyrophilin (band 12 protein) from bovine MFGM, seen with epifluorescence (*a* and *c*) or phase-contrast (*b*) optics. Note specific staining of alveolar epithelial cells and certain particles, probably milk fat globules, present in the alveolar lumina (*L*). The *inset* shows, in a slightly oblique section, staining of apical regions and of a budding milk fat globule (arrow). The restriction of immunofluorescence staining to the apical region is especially well seen, at higher magnification, in oblique sections (*b* and *c*). Note generally high phase contrast, i.e., dry mass concentration, in apical regions (e.g., *b*), probably related to high density of proteinaceous structures in this region (cf. reference 13). Bars: *a*, 100 μm; *inset* (in *a*), *b*, and *c*, 50 μm.

filaments are most commonly observed (13). Staining with antibodies to actin was not seen over lipid globules or over luminally disposed areas of budding lipid globules (Fig. 6*f*), 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. 6*f*, *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.[2] By contrast, antibodies to cytochrome b_5 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-

[2] W. W. Franke, C. Grund, and T. D. Keenan. Manuscript in preparation.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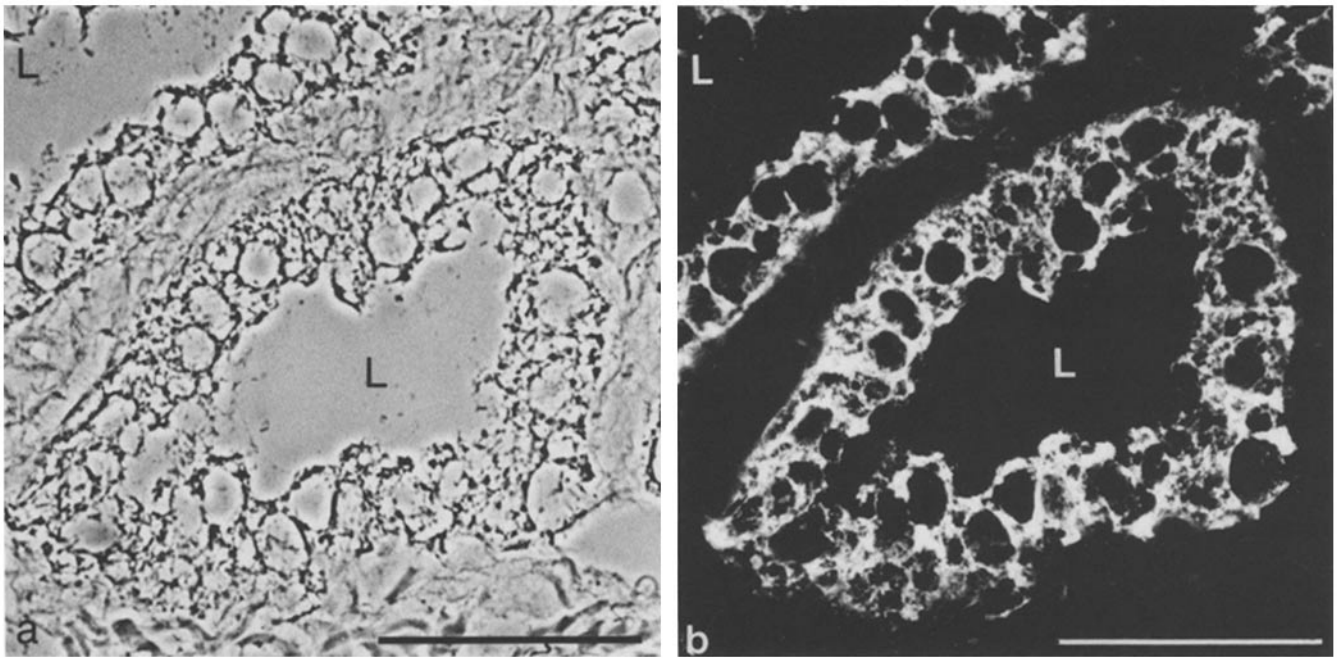

FIGURE 7 Immunofluorescence microscopy of frozen section through lactating bovine mammary gland as seen after staining with antibodies to cytochrome b_5. Note that the whole cytoplasm is intensely decorated by these antibodies, indicating that the endomembranes are very accessible to the IgGs. *a*, Phase contrast; *b*, epifluorescence. *L*, alveolar lumina. Bars, 50 μm.

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 M_r 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 distiguish 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 microscope 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. 9 *a*). As indicated by the *inset* to Fig. 9 *a*, 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. 9 *b* and *c*): strong staining was only seen on apical plasma membrane including surfaces of nascent milk fat globules (Fig. 9 *b*). No significant staining was detected on basolateral plasma membrane regions, in all other intracellular membranes, or in nuclei (Fig. 9 *b*). Negative reaction of butyrophilin antibodies was also observed in myoepithelial cells (Fig. 9 *b*) 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

FIGURE 6 Immunofluorescence microscopy of frozen sections through lactating mammary gland of cow after treatment with antibodies to butyrophilin from bovine MFGM (*a* and *b*), with guinea pig preimmune serum (*c*), with antibodies to prekeratin from desmosome-attached tonofilaments of bovine muzzle (*d*; antibody preparation GP_{19BET}), and with rabbit antibodies to actin (*e* and *f*). Note that antibodies to butyrophilin stain only apical regions of milk-secreting cells, including the surfaces of milk lipid globules that are in the process of budding (arrow in *a*) or detached (arrow in *b*) in the alveolar lumina (*L*). No staining is seen with control IgG (*c*). The staining pattern of antibodies to prekeratin is different and shows preference for myoepithelial cells (14) and desmosome-tonofilament complexes at cell boundaries (arrows in *d*). Antibodies to actin also stain, with special intensity, myoepithelial cells (e.g., *ME in insets* of *e* and *f*) and certain apical regions (e.g., arrows in *e* and *f*); they do not stain the surfaces of milk fat globules (*FG*) (*e*, phase contrast; *f*, epifluorescence) but often show strong reaction at basal regions of milk fat globule buds (e.g., arrows in *insets* of *e* and *f*). Bars, 25 μm (a, b, d, and *insets* in *e* and *f*) and 50 μm (*c*, *e*, and *f*).

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 b_5 (Fig. 7; see also reference 8).

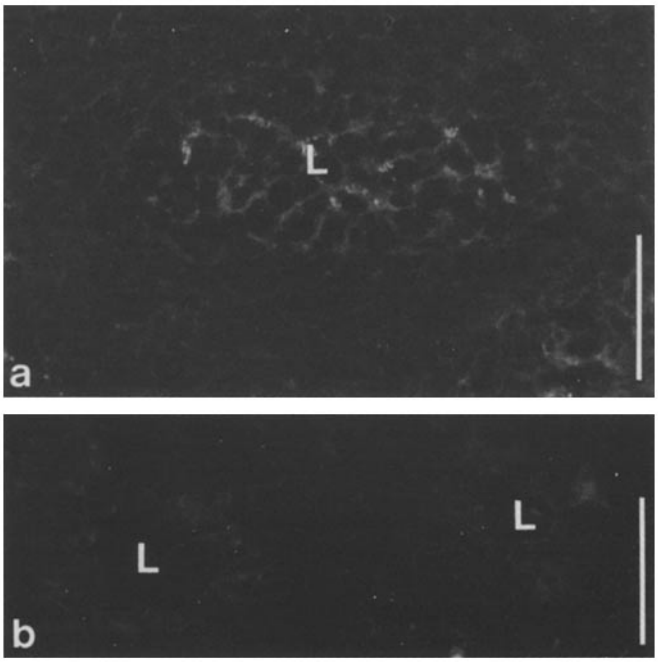

FIGURE 8 Immunofluorescence microscopy of frozen sections through bovine small intestine as seen in oblique (*a*) and cross (*b*) sections of intestinal villi after reaction with antibodies to band 12 protein of bovine MFGM. Note absence of significant staining. Bars, 25 μm.

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 membrane (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 nondenaturing 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 is 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.

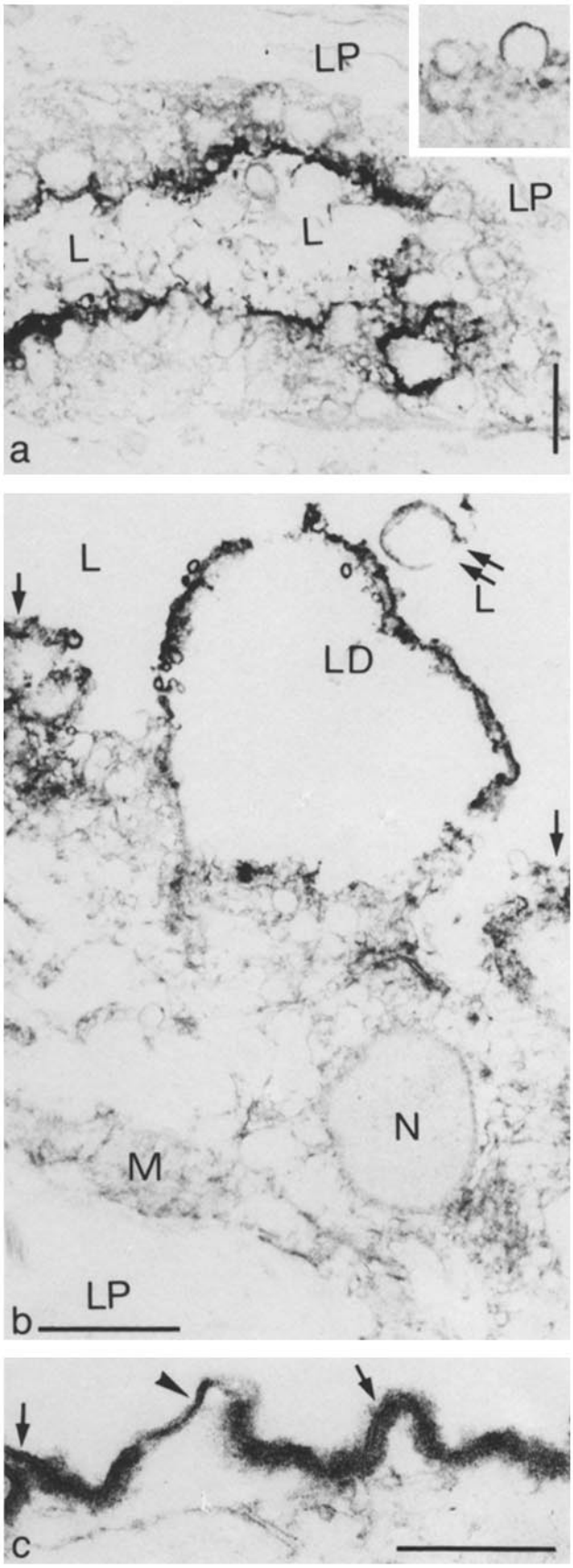

FIGURE 9 Light microscopy (*a*) and electron microscopy (*b* and *c*) of sections through frozen and dried tissue of cow's udder processed for localization of butyrophilin by the immunoperoxidase method using guinea pig antibodies (purified IgG) to butyrophilin from bovine MFGM. Light microscopy (*a*, bright field) of the immunoperoxidase reaction gives the same result as described for immunofluorescence microscopy and shows strong staining at the apical surfaces of milk-secreting epithelial cells, including surfaces of milk fat globules (*inset* in *a*) budding into the alveolar lumen (*L*). No significant staining is seen in cells of the lamina propria (*LP*). Electron microscopy of ultrathin sections through such preparations (*b*) also shows an intensely stained apical rim that includes the apical hemisphere of budding milk lipid droplets (*LD*). The arrows (*b*) indicate regions of apical cell cortex included in a slightly oblique section; the double arrow denotes a positively stained delaminated fragment of MFGM lying free in the alveolar lumen (*L*). *N*, nucleus of milk-secreting cell; *M*, myoepithelial cell; *LP*, lamina propria. Higher magnification (*c*) of the periphery of budding milk fat globules shows positive immunoreaction in the whole coat structure covering the inner aspect of the surface membrane (the membrane structure proper is lost in large parts as a result of the preparation involving feeezing, thawing, air-drying, and treatment with acetone and a series of buffer solutions, before fixation with aldehydes). Arrows (*c*) denote regions showing "unit membrane" structure; the arrowhead denotes a small membrane region apparently devoid of internal coat. Bars: *a*, 20 μm; *b*, 5 μm. *a* and *inset*, × 650; *b*, × 4,800; *c*, × 46,000.

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REFERENCES

1. Anderson, N. L., and B. J. Hickman. 1979. Analytical techniques for cell fractions. XXIV. Isoelectric point standards for two-dimensional electrophoresis. *Anal. Biochem.* 83:189–193.
2. Bargmann, W., and A. Knoop. 1959. Über die Morphologie der Milchsekretion. Licht- und elektronenmikroskopische Studien an der Milchdrüse der Ratte. *Z. Zellforsch. Mikrosk. Anat.* 53:344–388.
3. Blomberg, F., R. S. Cohen, and P. Siekevitz. 1977. The structure of postsynaptic densities isolated from dog cerebral cortex. II. Characterization and arrangement of some of the major proteins within the structure. *J. Cell Biol.* 74:204–225.
4. Bommeli, W. 1978. Die Ultrastruktur der Milchdrüsenalveole des Rindes, insbesondere die Basalfalten des Epithels und der Mitochondrien-Desmosomen-Komplex. *Zentralbl. Veterinaermed. Reihe C.* 1:299–325.
5. Bretscher, A., and K. Weber. 1978. Localization of actin and microfilament-associated proteins in the microvilli and terminal web of the intestinal brush border by immunofluorescence microscopy. *J. Cell Biol.* 79:839–845.
6. Bretscher, A., and K. Weber. 1979. Villin: the major microfilament-associated protein of the intestinal microvillus. *Proc. Natl. Acad. Sci. U. S. A.* 76:2321–2325.
7. Bretscher, A., and K. Weber. 1980. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335–340.
8. Bruder, G., A. Fink, and E. D. Jarasch. 1978. The *b*-type cytochrome in endoplasmic reticulum of mammary gland epithelium and milk fat globule membranes consists of two components, cytochrome b_5 and cytochrome P-420. *Exp. Cell Res.* 117:207–217.
9. Craiz, S. W., and J. V. Pardo. 1979. Alpha-actinin localization in the junctional complex of intestinal epithelial cells. *J. Cell Biol.* 80:203–210.
10. Danno, G.-I. 1977. Isoelectric focusing of proteins separated by SDS-polyacrylamide gel electrophoresis. *Anal. Biochem.* 83:189–193.
11. Franke, W. W., B. Appelhans, E. Schmid, C. Freudenstein, M. Osborn, and K. Weber. 1979. The organization of cytokeratin filaments in the intestinal epithelium. *Eur. J. Cell Biol.* 19:255–268.
12. Franke, W. W., C. Grund, E. Schmid, and E. Mandelkow. 1978. Paracrystalline arrays of membrane-to-membrane cross bridges associated with the inner surface of plasma membrane. *J. Cell Biol.* 77:323–328.
13. Franke, W. W., M. Lüder, J. Kartenbeck, H. Zerban, and T. W. Keenan. 1976. Involvement of vesicle coat material in casein secretion and surface regeneration. *J. Cell Biol.* 69:173–195.
14. Franke, W. W., E. Schmid, C. Freudenstein, B. Appelhans, M. Osborn, K. Weber, and T. W. Keenan. 1980. Intermediate-sized filaments of the prekeratin type in myoepithelial cells. *J. Cell Biol.* 84:633–654.
15. Franke, W. W., E. Schmid, J. Kartenbeck, D. Meyer, H. J. Hacker, P. Bannasch, M. Osborn, K. Weber, H. Denk, J. C. Wanson, and P. Drochmans. 1979. Characterization of the intermediate sized filaments in liver cells by immunofluorescence and electron microscopy. *Biol. Cell.* 34:99–110.
16. Franke, W. W., E. Schmid, M. Osborn, and K. Weber. 1979. The intermediate-sized filaments of human endothelial cells. *J. Cell Biol.* 81:570–580.
17. Franke, W. W., E. Schmid, J. Vandekerckhove, and K. Weber. 1980. A permanently proliferating rat vascular smooth muscle cell with maintained expression of smooth muscle characteristics, including actin of the vascular smooth muscle type. *J. Cell Biol.* 87:594–600.
18. Franke, W. W., K. Weber, M. Osborn, E. Schmid, and C. Freudenstein. 1978. Antibody to prekeratin. Decoration of tonofilament-like arrays in various cells of epithelial character.

Exp. Cell Res. 116:429–445.
19. Freudenstein, C. 1979. Isolierung, Charakterisierung und Lokalisierung von Plasmamembran-assoziierten unlöslichen Proteinstrukturen. PhD Thesis, University of Heidelberg, Federal Republic of Germany. 106 pp.
20. Freudenstein, C., T. W. Keenan, W. N. Eigel, M. Sasaki, J. Stadler, and W. W. Franke. 1979. Preparation and characterization of the inner coat material associated with fat globule membranes from bovine and human milk. *Exp. Cell Res.* 118:277–294.
21. Geiger, B. K., T. Tokuyasu, and S. J. Singer. 1979. Immunocytochemical localization of α-actinin in intestinal epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* 76:2833–2837.
22. Granger, B. L., and E. Lazarides. 1979. Desmin and vimentin coexist at the periphery of the myofibril Z disc. *Cell.* 18:1053–1063.
23. Helminen, H. J., and J. L. E. Ericsson. 1968. Studies on mammary gland involution. I. On the ultrastructure of the lactating mammary gland. *J. Ultrastruct. Res.* 25:193–213.
24. Huggins, J. W., T. P. Trenbeath, R. W. Chesnut, C. A. C. Carraway, and K. L. Carraway. 1980. Purification of plasma membranes from rat mammary gland. *Exp. Cell Res.* 126: 279–288.
25. Jarasch, E. D., G. Bruder, T. W. Keenan, and W. W. Franke. 1977. Redox constituents in milk fat globule membranes and rough endoplasmic reticulum from lactating mammary gland. *J. Cell Biol.* 73:223–241.
26. Keenan, T. W., W. W. Franke, I. H. Mather, and D. J. Morré. 1978. Endomembrane composition and function in milk formation. *In* Lactation. B. A. Larson, editor. Academic Press, Inc., New York. 4:405–436.
27. Keenan, T. W., C. Freudenstein, and W. W. Franke. 1977. Membranes of mammary gland. XIII. A lipoprotein complex derived from bovine milk fat globule membrane with preparative characteristics resembling those of actin. *Cytobiologie* 14:259–278.
28. Keenan, T. W., M. Sasaki, W. N. Eigel, D. J. Morré, W. W. Franke, I. M. Zulak, and A. A. Bushway. 1979. Characterization of a secretory vesicle-rich fraction from lactating bovine mammary gland. *Exp. Cell Res.* 124:47–61.
29. Kelly, P. T., and C. W. Cotman. 1978. Synaptic proteins. Characterization of tubulin and actin and identification of a distinct polysynaptic density polypeptide. *J. Cell Biol.* 79:173–183.
30. Krohne, G., and W. W. Franke. 1980. Immunological identification and localization of the predominant nuclear protein of the amphibian oocyte nucleus. *Proc. Natl. Acad. Sci. U. S. A.* 77:1034–1038.
31. Mather, I. H. 1978. Separation of the proteins of bovine milk fat globule membrane by electrofocusing. *Biochim. Biophys. Acta.* 514:25–36.
32. Mather, I. H., and T. W. Keenan. 1975. Studies on the structure of milk fat globule membrane. *J. Membr. Biol.* 21:65–85.
33. Mather, I. H., K. Weber, and T. W. Keenan. 1977. Membranes of mammary gland. XII. Loosely associated proteins and compositional heterogeneity of bovine milk fat globule membrane. *J. Dairy Sci.* 60:394–402.
34. Murray, L. R., K. M. Powell, M. Sasaki, W. N. Eigel, and T. W. Keenan. 1979. Comparison of lectin receptor and coat associated glycoproteins by milk lipid globule membranes. *Comp. Biochem. Physiol. B Comp. Biochem.* 63:137–145.
35. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 25:4007–4021.
36. Osborn, M., R. E. Webster, and K. Weber. 1978. Individual microtubules viewed by immunofluorescence and electron microscopy in the same PtK_2 cell. *J. Cell Biol.* 77:R27–R34.
37. Patton, S., and T. W. Keenan. 1975. The milk fat globule membrane. *Biochim. Biophys. Acta.* 415:273–309.
38. Peixoto de Menezes, A., and P. Pinto da Silva. 1978. Freeze-fracture observations of the lactating rat mammary gland. *J. Cell Biol.* 76:767–778.
39. Powell, J. T., U. Järlfors, and K. Brew. 1977. Enzymatic characteristics of fat globule membranes from bovine colostrum and bovine milk. *J. Cell Biol.* 72:617–627.
40. Ramaekers, F. C. S., M. Osborn, E. Schmid, K. Weber, H. Bloemendal, and W. W. Franke. 1980. Identification of the cytoskeletal proteins in lens-forming cells, a special epithelioid cell type. *Exp. Cell Res.* 127:309–327.
41. Sasaki, M., W. N. Eigel, and T. W. Keenan. 1978. Lactose and major milk proteins are present in secretory vesicles from mammary gland. *Proc. Natl. Acad. Sci. U. S. A.* 75: 5020–5024.
42. Sun, T. T., C. Shih, and H. Green. 1979. Keratin cytoskeletons in epithelial cells of internal organs. *Proc. Natl. Acad. Sci. U. S. A.* 76:2813–2817.
43. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76:4350–4354.
44. Wooding, F. B. P. 1971. The mechanism of secretion of the milk fat globule. *J. Cell Sci.* 9:805–821.
45. Wooding, F. B. P. 1971. The structure of the milk fat globule membrane. *J. Ultrastruct. Res.* 37:388–400.
46. Wooding, F. B. P. 1973. Formation of the milk fat globule membrane without participation of the plasmalemma. *J. Cell Sci.* 13:221–235.
47. Yen, S., P. Dahl, M. Schachner, and M. L. Shelanski. 1976. Biochemistry of the filaments of brain. *Proc. Natl. Acad. Sci. U. S. A.* 73:529–533.
48. Zerban, H., and W. W. Franke. 1977. Structures indicative of keratinization in lactating cells of bovine mammary gland. *Differentiation.* 7:127–131.
49. Zerban, H., and W. W. Franke. 1978. Milk fat globule membrane devoid of intramembranous particles. *Cell Biol. Int. Rep.* 2:87–98.