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130 words

Immunocytochemical Evidence for Golgi Vesicle Involvement in Milk Fat Globule Secretion

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Short title: Golgi vesicle role in Milk Fat Globule secretion

Abstract

The exact mechanism of secretion of the milk fat globule (MFGM) from the mammary secretory cell is still controversial. We have previously suggested close involvement of Golgi vesicles in this process. This paper provides direct immunocytochemical evidence that butyrophilin is present in the Golgi stack and vesicles in ovine and caprine mammary glands. We suggest that it is the butyrophilin in the golgi vesicle membrane which forms the specific association with the adipophilin on the lipid surface in the cytoplasm. Exocytosis of the associated Golgi vesicle will then initiate the process of MFG secretion. Further exocytosis of associated Golgi vesicles will continue and complete the process. Areas of the plasmalemma which have butyrophilin delivered by previous non lipid associated Golgi exocytoses may also contribute to the process of forming the MFGM.

130 words

Introduction

Despite numerous investigations over many years there is still no consensus on the mechanism of secretion of the milk fat globule [MFG] nor the structure of the milk fat globule membrane [MFGM] after secretion. The individual proteins and phospholipids involved have been comprehensively investigated and butyrophilin [BTN], xanthine oxidase [XO], adipophilin [ADP/Plin2]are generally accepted as the building blocks of the MFGM but the ways in which they interact to form the MFGM are still controversial. (McManaman et al. 2002; Heid and Keenan 2005; Chong et al. 2011; Mather 2011).

We have recently demonstrated that the biochemical structure of BTN is consistent with processing via the Golgi system (Jeong et al. 2013). This would support the electron microscopical observations of Wooding (1971, 1977) that in all species examined a significant area of Golgi secretory vesicle membrane associates with the perimeter of the intracellular apical lipid droplet to preform patches of a structure identical to the MFGM around the newly secreted, alveolar MFG.

This general observation was reinforced by finding that, under certain conditions in the goat mammary gland, the MFGM could form intracellularly by fusion of the lipid associated Golgi vesicles with each other without participation of the apical plasmalemma (Wooding 1975).This produced a large intracellular vacuole containing a MFG bounded by a MFGM.

The conventional fusion of the Golgi vesicles with the plasma membrane would allow the "patches" of membrane association with the lipid to aggregate into the continuous unit membrane separated from the lipid by a uniform 15nm of material continuous with the cytoplasm.

If two Golgi vesicles separated by a cytoplasmic "bleb" fused simultaneously with the plasma membrane then this would produce the "signet ring" MFGM which are found in a small variable percentage in all milks so far investigated (Wooding 1977).

This scenario takes into account all the EM observations on a variety of species: cow, ewe, goat and rodents as long as the material is fixed by aldehyde perfusion as soon as practical after the death of the animal. Any fixation can produce artefacts but if the perfusion produces uniform fixation with resolvable membranes the results are always more reliable than with immersion fixation. Immersion fixation must produce a variety of fixation quality from edge to middle of the block and this seems especially true of lipid laden tissue. Immersion fixation can produce useful corroborative evidence but any micrographs

from either fixation must show resolvable unit membranes if used to support a mechanism for MFGM formation.

Butyrophilin has previously been localised with immunocytochemistry to the apical plasmalemma of the mammary cell in several mammals and around the MFG in the alveolus (Mather and Jack 1990, 1993; Heid and Keenan 2005) but no one has been able to demonstrate any cytoplasmic label indicating the route to the plasmalemma.

We now have more direct evidence for the involvement of the Golgi vesicles with butyrophilin synthesis and transport using Light Microscope (LM) immunocytochemistry. Using an antibody raised in rabbits against a mouse butyrophilin peptide on sections of perfused sheep or goat mammary gland we get an identical supranuclear localisation to localisation produced by a rabbit anticasein antibody (Uejyo et al. 2015). Casein is generally accepted to be processed in the Golgi and secreted via the Golgi vesicles (Linzell and Peaker 1971; Beery et al 1971: Wooding 1977: Mather 2011). Other butyrophilin antibodies sometimes show indications of such a localisation but never show such reproduceability and uniformity.

Materials and methods

Electron Microscopy

Mammary tissue from 4 lactating goats, 5 ewes and 2 cows was either excised immediately after death by barbiturate overdose and cut into small cubes in the glutaraldehyde fixative or fixed initially by perfusion via the mammary artery (goat, ewes). All the processing was carried out at room temperature

All animal work was carried out in accordance with the Animals(Scientific Procedures) Act 1986.

The fixation was in 4% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, containing 2% sucrose, for 45 min. The tissue was washed briefly in buffer, postfixed first in 1 % osmium

tetroxide in 0.1M veronal buffer, pH 7-2, for 30 min, then in 5 % aqueous uranyl acetate for 2 h followed by ethanol

dehydration and embedding in Araldite. Sections were cut on an LKB Ultrotome, stained with uranyl acetate and lead hydroxide and observed in a AEI EM6B microscope operated at 60 kV.

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Light Microscope Immunocytochemistry.

Tissue was fixed by immersion or perfusion in 4% formaldehyde in 0.1M phosphate buffer, pH 7-2, or in 4% formaldehyde plus 1% glutaraldehyde in 0.1M phosphate buffer, pH 7-2. No Osmium was used.

Dehydration and embedding was at room temperature for araldite resin. Semi-thin 1-2µm sections were cut from the araldite blocks and picked up on coverglass squares coated with APES . Resin was removed from semithin araldite sections by incubation in sodium ethoxide solution (15 grams of sodium hydroxide pellets dissolved in 15 mls of absolute alcohol) for 15 minutes followed by alcohol and water washes.

For immunocytochemistry the coverslip squares were floated section side down on drops of antibody followed by immunogold colloid [Goat anti rabbit G4, Jackson Immunoresearch Labs, USA], then intensified with silver reagent [Aurion, Wageningen, Netherlands] followed if necessary by 0.05% fast green counterstain.

The following primary antibodies were used: rabbit anti beta casein (ab91167, Abcam, 1:3000), rabbit anti MCT-1 (Halestrap and Price, 1999, 1:100), rabbit anti Glucose transporter 1 (Baldwin et al, 1982, 1:1000), rabbit anti Lactoferrin (ab15811, Abcam, 1:100), Rabbit anti CD36, 1:100 (a gift from Prof M Febbraio (see Silverstein and Febbraio 2009).

Three antibodies from Prof. I Mather: rabbit anti butyrophilin 1A1 (bovine whole molecule, 1:1000), rabbit anti butyrophilin1A1 (peptide from mouse butyrophilin,

1:1000) and goat anti adipophilin (Plin 2) (1:3000), see Mather 2000 for references.

Two GM 130 antibodies (cis Golgi body marker): Mouse anti GM130 (610822,BD Transduction Labs) and Rabbit anti GM 130 (ab52649,Abcam). Antibodies used with or without prior Heat induced antigen retrieval (sections treated 3x2mins at 95^{O} in 0.1M NaCitrate buffer, pH6).

Immunocytochemical controls, in which the primary antibody was omitted and replaced with buffer or a non specific antibody at the same concentration, were carried out routinely, alongside the experimental samples. Controls showed an insignificant level of labelling, see Figure 2d.

Western Blotting

Mammary tissue from a ewe in full lactation was sampled and immediately frozen in liquid nitrogen. Milk from the same ewe was spun to concentrate the cream which was then resuspended in 50 times by volume phosphate buffered saline at 37^{0}C, respun, and the washed cream was frozen in liquid nitrogen.

Mammary tissue and cream were ground to a fine powder using a mortar and pestle. Protein from mammary gland or cream powder was extracted in RIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA and 1× Complete protease inhibitor (Roche), 1 mM Na_3VO_4, and 1 mM NaF). Protein concentration was assessed with the BCA Protein Assay (Thermo Fisher Scientific) and equal amounts of protein (20 µg) were denatured and resolved on SDS–polyacrylamide gels. Immunoblotting was performed using standard techniques and antibody detection was achieved with enhanced chemiluminescence reagent (ECL, GE Healthcare). The primary antibody used was rabbit anti- BTN 1A1 (a gift from I. Mather, 1:5,000).

Results

The uniformity of the ultrastructure of milk secretion involving Golgi vesicle association with the cytoplasmic lipid has been clearly shown previously (Wooding 1977) and is reinforced by Fig 1a from ewe mammary gland. All the five apical lipid droplets are surrounded by rosettes of Golgi vesicles. Figs 1b-f illustrate in a variety of species the consistent formation of a flattened area of Golgi vesicle membrane paralleling the lipid contour. The unit membrane is separated only by a 100-150 angstrom cytoplasmic gap from the single line of electron dense, probably lipoprotein, material around the lipid globule. Fig 1g shows the structure of the Milk Fat Globule Membrane [MFGM] immediately after secretion, this would be the structure of the alveolar facing membrane around the partly secreted lipid in Fig 1h.

The casein containing Golgi vesicles are programmed to fuse with the apical plasma membrane as seen, for example, in Fig 1f. Continued exocytosis of the adjacent lipid associated vesicles in 1f would form a nascent MFGM facing the alveolus leading eventually to the lipid droplet in Fig 1h with its associated Golgi vesicles ideally positioned to exocytose and release the MFG into the alveolus.

What is the basis for the association between the Golgi vesicles and the lipid ? One of the components of the lipoprotein boundary around the lipid in the mammary cell and alveolus is adipophilin (Plin 2) as can be seen in Fig 3e. Figs 3c, 4, 5 and 6 show in ewe and goat that the Golgi area and vesicles can be labelled with an antibody to butyrophilin [BTN]. The specificity of this antibody is confirmed by the western blot results on Fig 8. The identification of the site of the BTN label as the Golgi area plus vesicles is confirmed by the similar localisation, on adjacent sections, of casein (Fig 3b) and lactoferrin (Fig 3d), proteins known to be processed via the Golgi (Linzell and Peaker 1971; Beery et al 1971; Wooding 1977). Also the supranuclear label and the label surrounding the lipid droplets is in the identical position to that shown on the Electron

Microscope for the mammary cell Golgi body and vesicles as can be seen on Figs 1a and h.

Unfortunately this Golgi localisation could not be additionally confirmed using two commercial antibodies to a Golgi protein, GM130, neither produced any localisation in this immunocytochemical system.

This BTN localisation can only be shown with a rabbit anti mouse BTN peptide, and is restricted to the ewe and goat mammary tissue. None of the other BTN antibodies show this localisation. Previous papers using rabbit anti-bovine BTN protein or peptide show a localisation only to the apical membrane and the alveolar MFG (Franke et al 1981). This is corroborated by our results with such a bovine antibody on adjacent sections to those using the anti mouse BTN in Figs 3, 4 and 6.

The immunocytochemical method used here, with deresinated 1-2μm sections is unusual but has been validated in a number of studies, see Groos et al 2001. To confirm its validity here, Figs 2 and 3 show that the localisations on adjacent sections of ewe mammary gland of glucose transporter 1, monocarboxylic acid transporter 1, CD36(Fatty acid transporter), adipophilin (Plin 2), casein and lactoferrin are no different from those shown previously (Uejyo et al 2015, Tachebe et al 2009) for the rodent and/or the bovine gland. The deresinated section method has the advantage in providing a much better resolution than the normal 5-10 um wax section. (see Figure 2 in Groos et al 2001).

Close examination of sections from mammary glands of Guinea Pig, Rat, Wildebeeste and Zebra showed no cytoplasmic localisation with the anti mouse BTN antibody [results not shown] although in Bovine and mouse there is occasional indication of label adjacent to apical cytoplasmic lipid (Figs 7a and b).

Preliminary investigation of mouse BTN antibody with EM immunocytochemistry of ewe mammary tissue showed similar indications of labelled Golgi vesicles but the membrane

definition is too poor and the deresinated thin sections too fragile to label and counterstain reliably.

Discussion

Our previous biochemical demonstration that butyrophilin [BTN] is processed through the Golgi in the mouse mammary cell (Jeong et al. 2013) is now reinforced by the immunocytochemical evidence in this paper that localises the BTN label for the first time to the area on sections which contains the Golgi and/or Golgi vesicles.

However this cytoplasmic localisation is unequivocal only with a specific antibody on two particular species, sheep and goat. Western blotting confirms that this antibody is recognising butyrophilin. In the cow and rodent mammary cells there are no clear indications of any cytoplasmic label. However with both BTN antibodies label is seen on the milk fat globule membrane [MFGM] around the secreted milk fat globules [MFG] in the alveolus and this is true of all species investigated so far. Label is also usual on the alveolar plasmalemma (Mather and Jack 1993; Heid and Keenan 2005).

This Golgi localisation gives a rationale for the clustering of Golgi vesicles around apical MFG observed in all species examined with the electron microscope so far (Wooding 1977). The association is strong enough to distort the initially spherical Golgi vesicle to form a localised flattened membrane area parallel to the MFG contour but always separated from it by a 15 nanometer gap which is continuous with the adjacent cytoplasm, see figure 1.

The direct involvement of the Golgi vesicles in both protein and lipid secretion is also supported by Patton's papers (Patton et al 1977; Knudson et al 1978) using infusions of colchicine into the teats of rats and goat. This treatment inhibits milk secretion by blocking Golgi vesicle exocytosis.

Butyrophilin has a transmembrane sequence and the C- terminal portion of the molecule would protrude into the cytoplasm from the Golgi vesicle (Mather and Jack 1990,1993; Jeong et al. 2013). Interaction of this C-terminal sequence with the adipophilin(Plin 2) present around the contour of the lipid and the xanthine oxidase from the cytoplasm could provide the force necessary for the formation of the Golgi –lipid association.

There is good biochemical evidence for a specific trimer formation between these three molecules in the MFGM (McManaman et al. 2002; Jeong et al. 2013) and this is the basis for several hypotheses for formation of the MFGM but with butyrophilin on the plasma membrane. Antibodies to the bovine butyrophilin label the mammary cell apical plasma membranes in several species. The mammary Golgi vesicles are designed to fuse with the apical plasma membrane whether or not they are associated with lipid so that some would deliver some BTN there directly, and some subsequently after associating with the apical lipid.

The different localisations with the different antibodies could be due to differences in epitopes and/or epitope availability. In sheep and goat with the anti mouse antibody the epitope is clearly available on the Golgi vesicles, weakly on the plasma membrane and readily on the alveolar MFGM. The bovine antibody does not bind until the BTN is exposed to the luminal environment so it is possible that the epitope is occupied with a cytoplasmic molecule. If interaction between the BTN, XO and ADP molecules is the basis for the formation of the MFGM this will potentially also affect epitope availability, especially since in the alveolus the MFGM in several species modifies to produce a quasicrystalline protein lattice in the space between membrane and lipid contour (Wooding 1977; Buchheim 1982,

1986, and Buchheim et al, 1988) hypothetically where the three proteins are located.

How necessary the Golgi vesicle association with the lipid in terms of total lipid secretion is difficult to judge. All species so far examined adequately in the EM show the close association and it clearly supplies the necessary components for formation of the MFGM. Also examples of exocytosis of a lipid associated Golgi vesicle to initiate, continue and complete secretion can readily be found on well fixed tissue in the EM (Wooding 1977; Kralj and Pipan 1992) whereas images of MFG being "enveloped" by the plasmalemma with no vesicles associated are in our experience far less frequent. It has been estimated (Mather 2011) that in the typical mammary cell far more membrane is available around Golgi vesicles than is required to envelop all lipid secreted by that cell. The results in this paper suggest that a significant amount of the MFGM is derived from Golgi vesicles directly associated with lipid prior to secretion.

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Declaration of Conflicting Interests

The authors declare no potential competing interests with respect to the research authorship and/or publication of this paper.

Authors Contribution

FBPW planned, discussed, carried out the immunocytochemistry and wrote the paper, TJS discussed, carried out the Western Blotting and corrected the paper.

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460 Figure Legends
461
462 **Fig.1** Electron Micrographs illustrating the Golgi vesicle - lipid
463 droplet association in mammary cells in full lactation. **a.** Ewe

mammary gland. All of the apical lipid cytoplasmic lipid droplets (1-5) show associated Golgi vesicles (arrowheads). Golgi bodies and their vesicles (ga) are extensive, the vesicles usually identified by their casein content. A, alveolus; c, capillary; L, basal lipid; n, nucleus of mammary cell; nm, nucleus of myoepithelial cell.

b(ewe), c(mouse), d(cow). Details of the Golgi vesicle – lipid association. This illustrates the uniformity of the cytoplasmic gap between the vesicle membrane and the lipid contour which is equivalent to the structure around the recently secreted alveolar Milk Fat Globule (MFG) seen on figure 1g.

e. Rat mammary gland. Typical Golgi vesicle – lipid association showing distortion of the Golgi vesicles to follow the contour of the lipid.

f. Rat mammary gland. Initiation of MFG secretion by exocytosis of a Golgi vesicle also associated with a cytoplasmic lipid droplet.

g. Horse mammary gland, high magnification of the structure of the recently secreted MFGM.

h. Ewe mammary gland. MFG nearly released, exocytosis of the remaining associated Golgi vesicles (arrowheads) at its base would continue and finally complete the process.

Scale bars: 5µm (a), 200nm (b,c,d,f), 2µm (e), 50nm (g),1µm (h)

Fig 2. Light Microscope (LM) immunocytochemistry of ewe mammary gland, adjacent sections of the same alveolus (A), the asterisk identifies different levels of the same capillary on each section**.**

a. Glucose transporter 1,

b. Monocarboxylic acid transporter 1, and

c. CD36/Fatty Acid Transporter all show the same localisation to the mammary cell basolateral plasmalemma (arrows).CD36 also shows capillary endothelium label (arrowheads).

d. Control, no antibody

Scale bar for all, 50µm.

Fig 3. Continuation of the LM immunocytochemistry of ewe mammary gland, same alveolus as Fig 2, same scale bar.

a. Butyrophilin localised with an antibody against bovine butyrophilin. Label is restricted to the alveolar plasmalemma (arrow) and the alveolar MFG (arrowheads).

b. Casein, localised to the Golgi area and around apical lipid drops (arrows), also to diffuse areas in the alveolus.

c. Butyrophilin localised with an antibody against mouse butyrophilin.Label is restricted to the Golgi area and around apical lipid drops (arrows). A few alveolar MFG are also labelled (arrowheads).

d. Lactoferrin is localised to the Golgi area and around apical lipid drops (arrows). The apical plasmalemma shows some label but alveolar MFG show none.

e. Adipophilin labels all the lipid droplet surfaces, both cytoplasmic (arrows) and alveolar (arrowheads).

519 **Fig 4.** Ewe mammary gland, closely adjacent sections of the
520 same alveolus (A), different levels of the same capillary are
521 marked by an asterisk. The 0.5-5μm white circular areas in the
522 cytoplasm of the mammary cells are lipid droplets, most obvious
523 in 4a.In the apical cytoplasm of cells in 4b, there are populations
524 of even smaller white circular areas (double arrowheads) which
525 by analogy with Fig 1a are most likely Golgi areas. This
526 conclusion is reinforced by the fact that similar areas on Figs 4a
527 (casein) and 4c (mouse butyrophilin) label positively. Arrows
528 denote the same lipid droplet in at least two of the sections.
529 Arrowheads in 4b and 4c indicate an MFG in the process of
530 secretion and an MFG free in the alveolus. The bovine
531 butyrophilin antibody labels only the apical plasmalemma but
532 the mouse antibody only labels the cytoplasmic structures.
533 Nuclei can occasionally be identified (curved arrows) Inset on
534 Fig 4a is the cell immediately below taken at x100, the
535 information available and the resolution are not significantly
536 improved. Scale bar same for all, 50μm.
537

538 **Fig 5.** Ewe mammary gland. Details of the mouse butyrophilin
539 antibody localisation, which by analogy with Fig 1a
540 (electron microscopy) and 4a (casein localisation) shows a
541 strong label in the apical cytoplasmic Golgi areas. The arrows
542 indicate lipid droplets surrounded by Golgi vesicles, arrowheads
543 indicate two MFG in the process of secretion and asterisks show
544 basal lipid droplets with no adjacent Golgi vesicles. sa, small
545 artery; c , capillary. Scale bar 8μm.
546
547 **Fig 6.** Goat mammary gland, closely adjacent sections of the
548 same alveolus (A), different levels of the same capillary are
549 marked by an asterisk. The micrographs show equivalent
550 localisation to Golgi areas as in the ewe with mouse
551 butyrophilin (6a) and casein (6c). With bovine butyrophilin (6b)
552 the cytoplasmic Golgi vesicles are free of label (arrowheads)
553 although the apical plasmalemma is heavily labelled. Neither
554 casein nor mouse butyrophilin produce any significant label on
555 the apical plasmalemma. Scale bar same for all, 50μm.
556

Fig 7. Cow (7a,b) and Mouse (7c) mammary glands. With the mouse anti butyrophilin antibody there are occasional indications (arrows) of label around or close to the apical lipid in cow (7b) and mouse (7c). However most sections show no significant cytoplasmic label, although the apical plasmalemma labels in the cow (7a). No label is found on the mouse sections with the bovine antibody (results not shown). Scale bars, 40μm (a,b), 50μm (c).

Fig 8. Western blot for Butyrophilin (M_r 70K) showing BTN present in both mammary tissue and cream samples.

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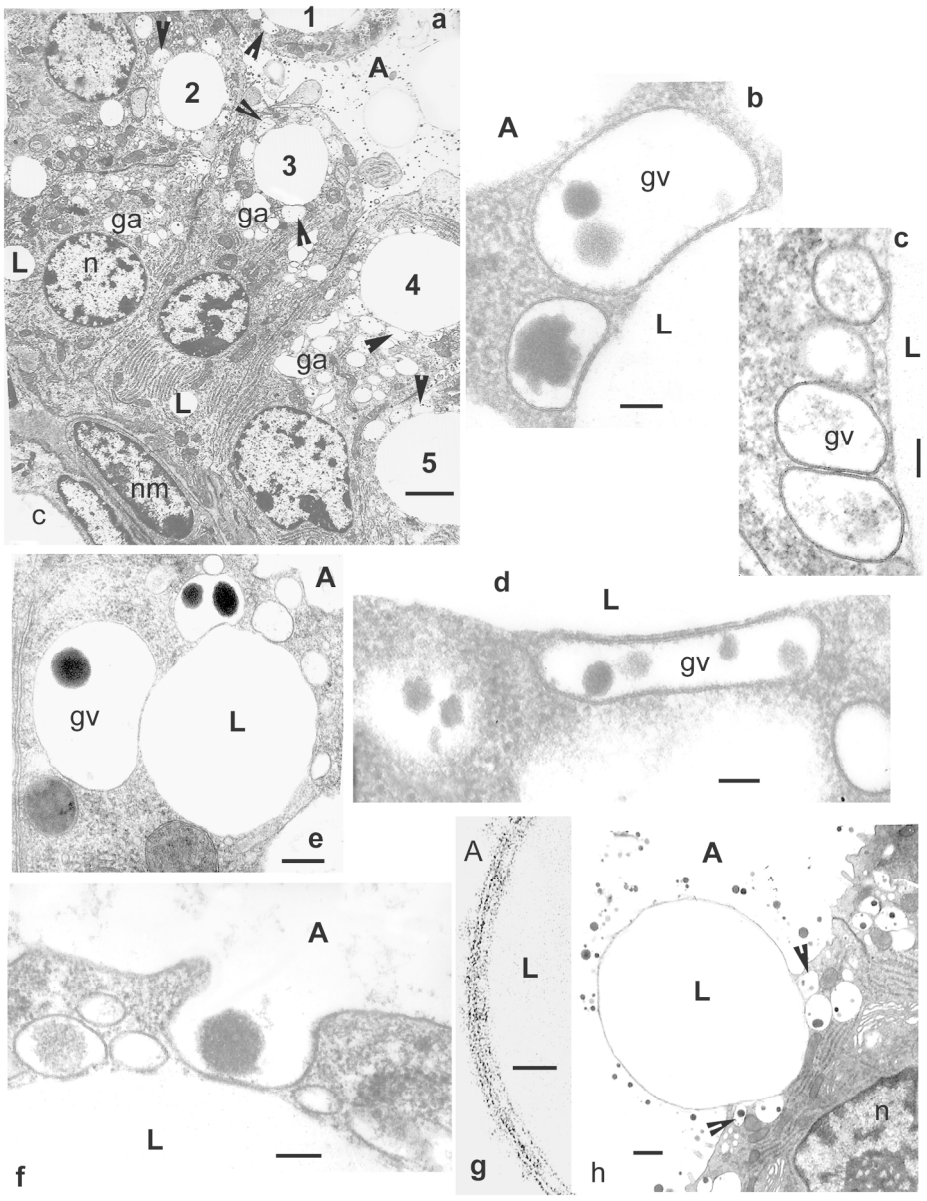

Figure 1
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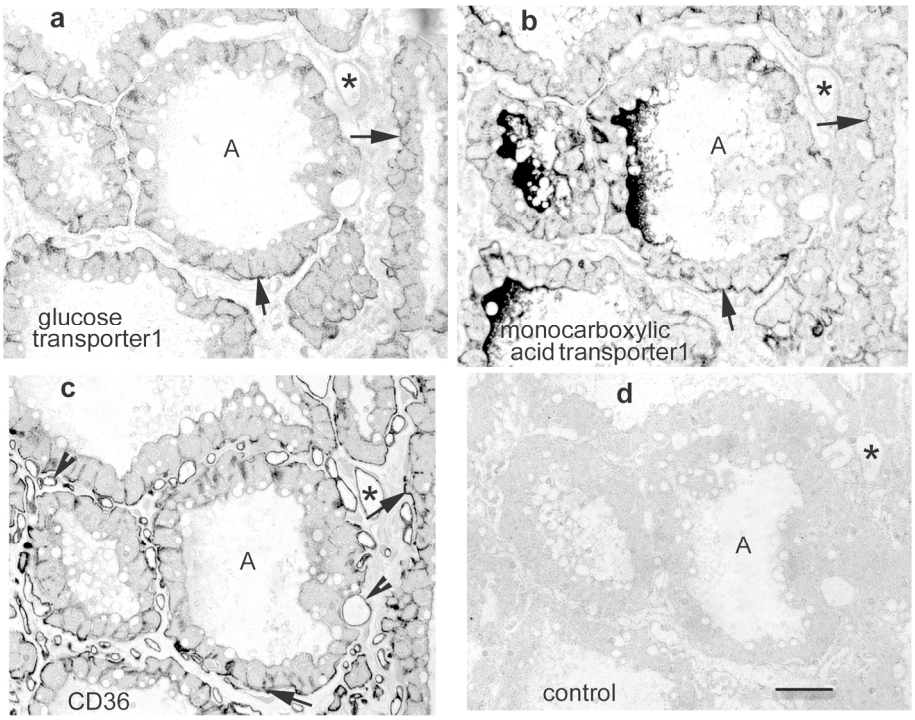

Figure 2
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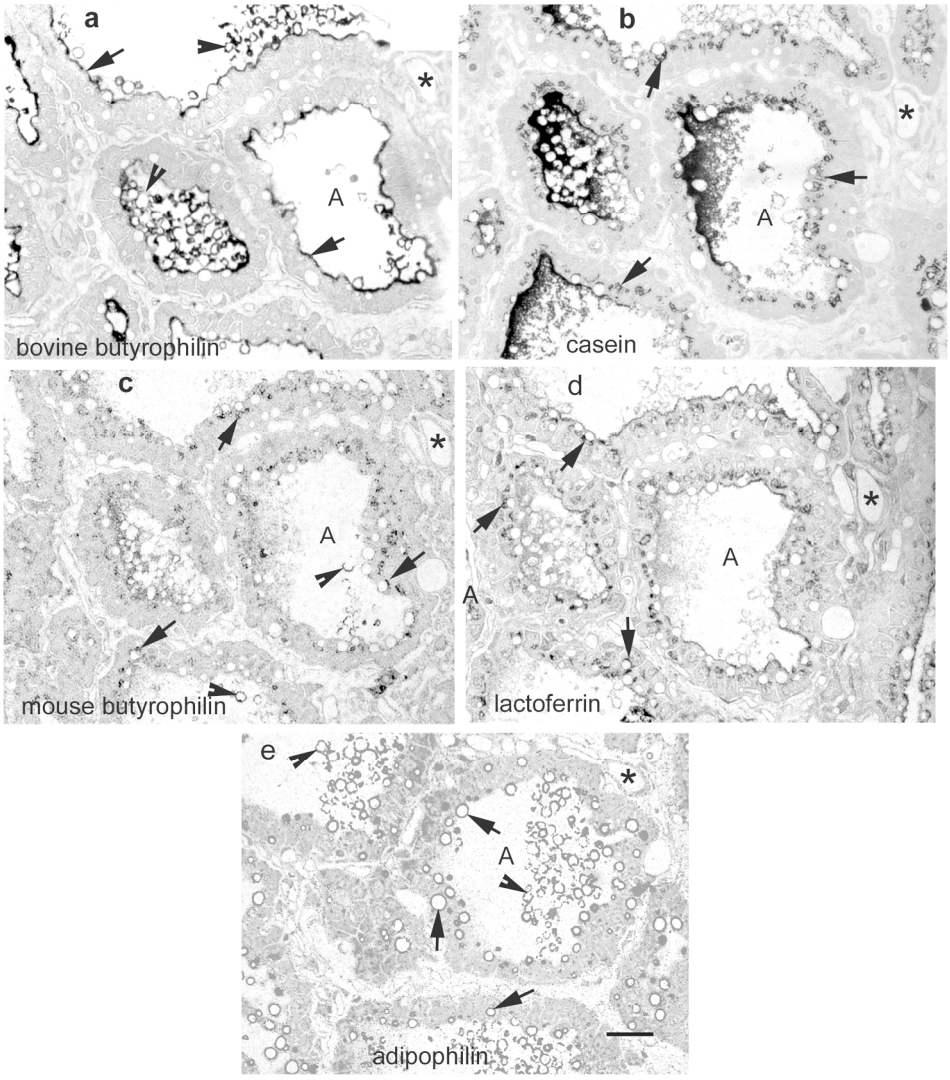

Figure 3
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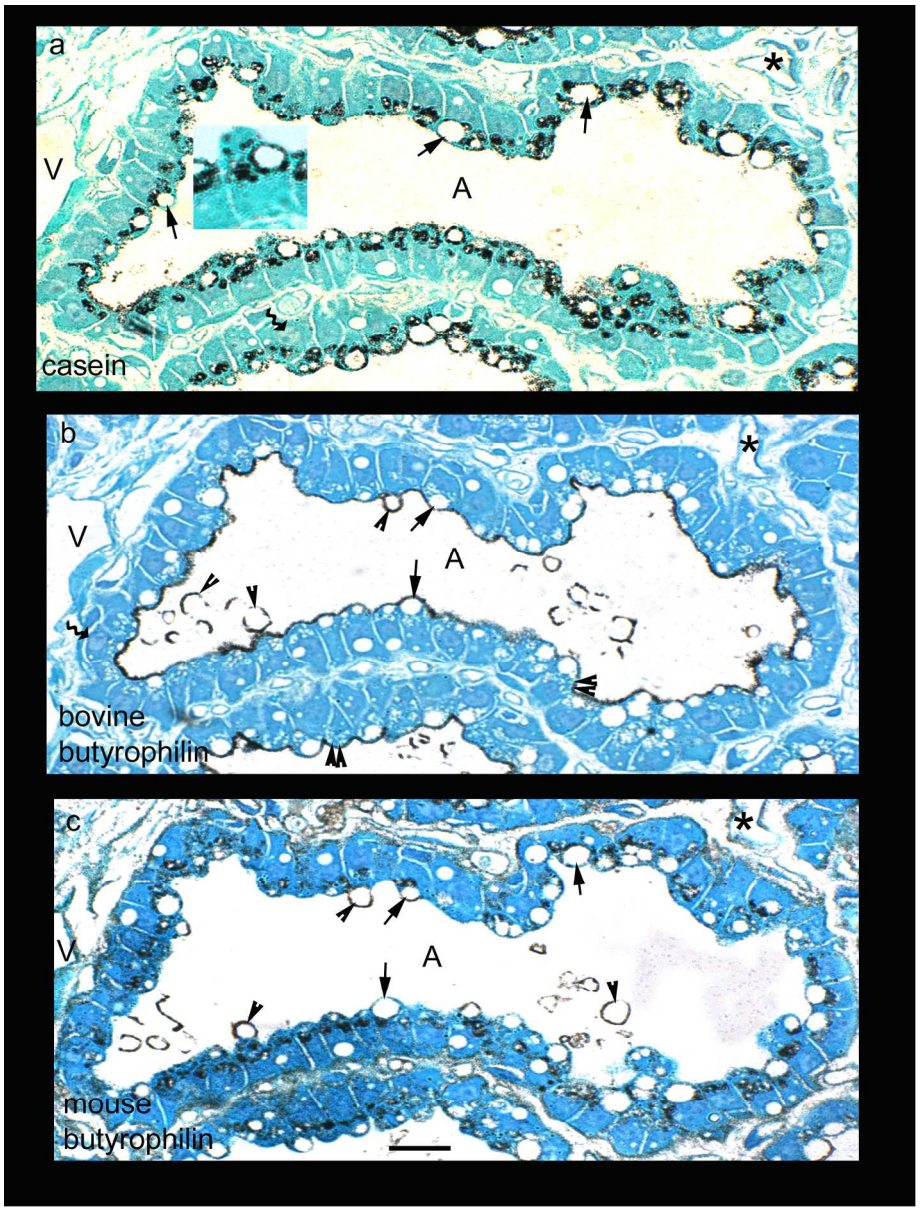
a
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butyrophilin

Figure 4

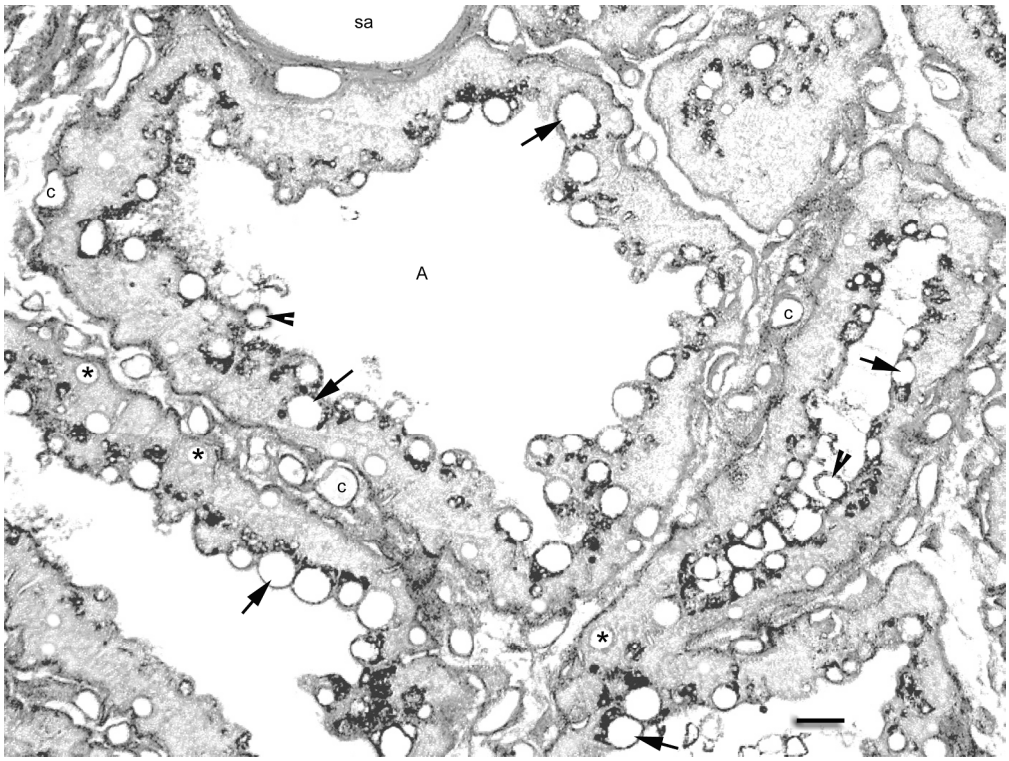

Figure 5
235x176mm (300 x 300 DPI)

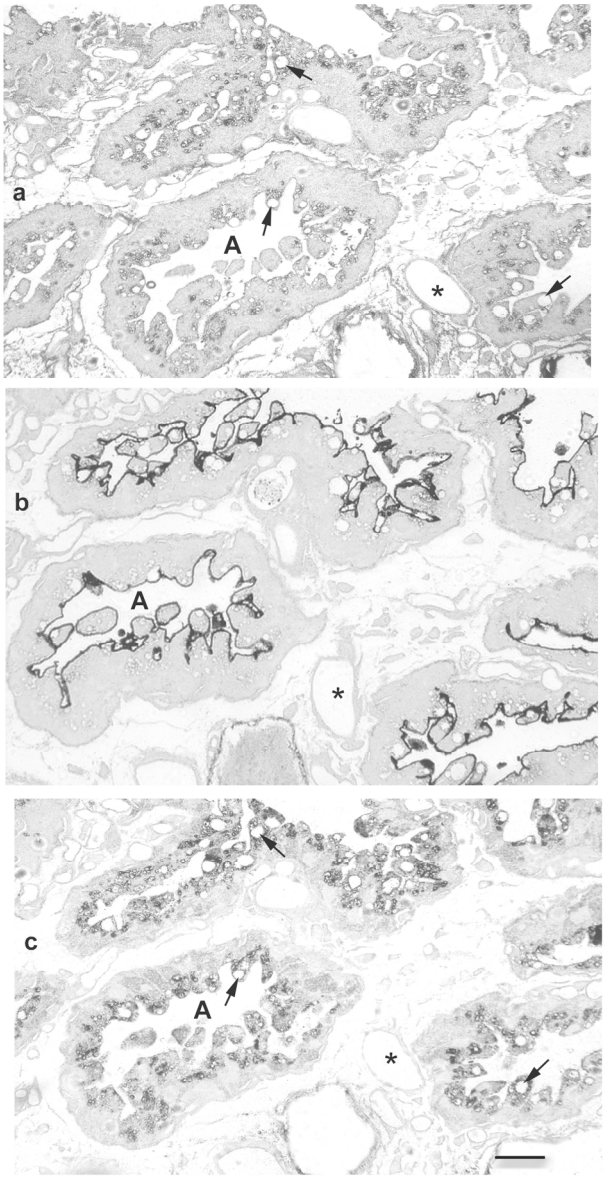

Figure 6
164x203mm (300 x 300 DPI)

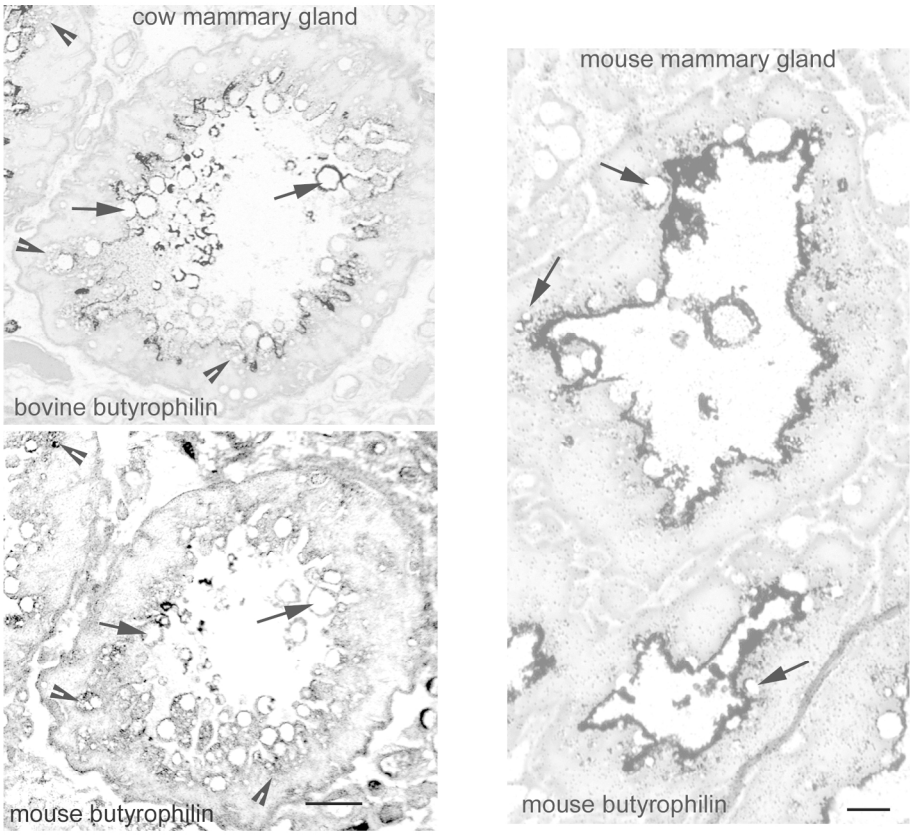

Figure 7
167x207mm (300 x 300 DPI)

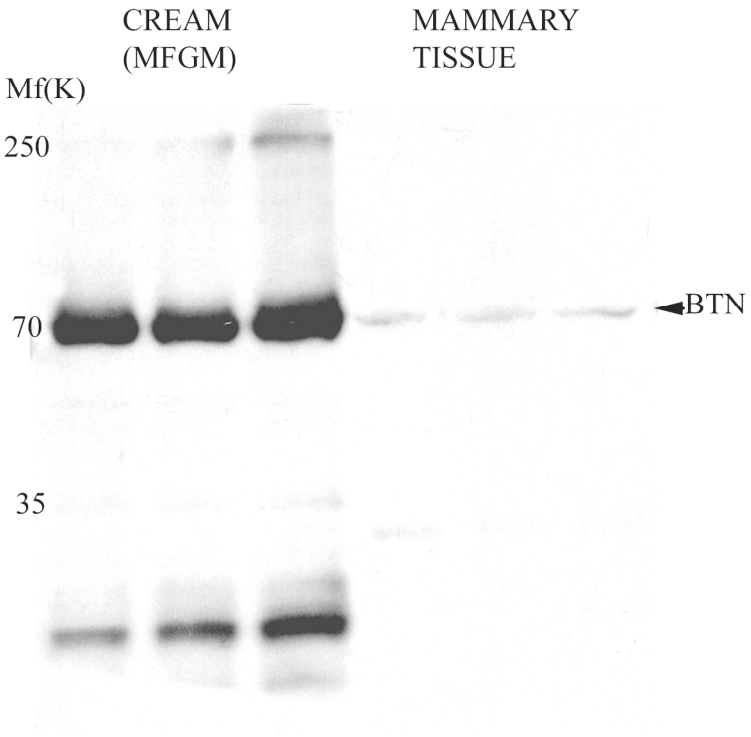

Figure 8