The lipid composition of autophagic vacuoles regulates expression of multilamellar bodies

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Summary

Multilamellar bodies (MLBs) are responsible for surfactant secretion in type II alveolar cells but also accumulate in other cell types under pathological conditions, including cancer and lysosomal storage diseases such as Niemann-Pick C (NPC), a congenital disease where defective cholesterol transport leads to its accumulation in lysosomes. Mv1Lu type II alveolar cells transfected with Golgi β1,6 N-acetylgalcosaminyltransferase V (Mgat5), enhancing the polylactosamine content of complex-type N-glycans, exhibit stable expression of MLBs whose formation requires lysosomal proteolysis within dense autophagic vacuoles. MLBs of Mgat5-transfected Mv1Lu cells are rich in phospholipids and have low levels of cholesterol. In Mv1Lu cells treated with the NPC-mimicking drug U18666A, cholesterol-rich MLBs accumulate independently of both Mgat5 expression and lysosomal proteolysis. Inhibition of autophagy by blocking the PI 3-kinase pathway with 3-methyladenine prevents MLB formation and results in the accumulation of non-lamellar, acidic lysosomal vacuoles. Treatment with 3-methyladenine inhibited the accumulation of monodansylcadaverine, a phospholipid-specific marker for autophagic vacuoles, but did not block endocytic access to the lysosomal vacuoles. Induction of autophagy via serum starvation resulted in an increased size of cholesterol-rich MLBs. Although expression of MLBs in the Mv1Lu cell line can be induced by modulating lysosomal cholesterol or protein glycosylation, an autophagic contribution of phospholipids is critical for the formation of concentric membrane lamellae within late lysosomal organelles.

Key words: Multilamellar bodies, Cholesterol, Lysosomes, Phosphatidylinositol 3-kinase, Autophagy, Autophagic vacuoles

Introduction

Multilamellar bodies are lysosomal organelles containing multiple concentric membrane layers. MLBs vary in size from 100 to 2400 nm and are found in various cell types where their principal functions are storage and secretion of lipids (Schmitt and Muller, 1991). In lung alveolar type II cells, MLBs are responsible for the secretion of the surfactant film that prevents alveolar collapse during respiration (Hatasa and Nakamura, 1965). Deficient expression of the hydrophobic surfactant protein B (SP-B) results in the formation of immature MLBs and secretion of non-functional surfactant (Foster et al., 2003). Abnormal MLBs have also been observed in familial desquamative and non-specific interstitial pneumonitis associated with mutations in surfactant protein C (SP-C) gene (Thomas et al., 2002). Another protein, the ATP-binding cassette transporter A3 (ABCA3) is also localized to lamellar bodies of alveolar type II cells and is critical for the formation of MLBs and production of surfactant (Mulugeta et al., 2002; Shulenin et al., 2004). MLB formation in type II alveolar cells is therefore critically dependent on the protein composition of the organelle.

The lipid composition of MLBs is 95% dipalmitoyl phosphatidylcholine (DPPC), a neutral phospholipid that represents the major active component of surfactant (Schmitz and Muller, 1991). Lung surfactant is composed of 80% glycerophospholipid, 10% cholesterol and 10% protein; the amount of cholesterol within the surfactant can increase relative to the phospholipid content under certain conditions, including exercise (Doyle et al., 1994) and hyperpnea (Orgeig et al., 2003). In lung MLBs, cholesterol is localized primarily to the limiting membrane of the organelle (Orgeig, 2001) and whether MLBs are the source of surfactant cholesterol remains uncertain (Hass and Longmore, 1979; Orgeig et al., 1995). Augmentation of cellular cholesterol stimulates MLB expression, the accumulation of cholesterol within MLBs and the uptake of palmitic acid, the precursor of DPPC, in alveolar type II cells (Kolleck et al., 2002). Extracellular cholesterol is therefore internalized and stored in the MLBs of type II alveolar cells but whether it impacts on their biogenesis remains poorly understood.

The presence of MLBs is also associated with various lysosomal storage diseases, including gangliosidosis, Tay-Sachs, Fabry’s and Niemann-Pick, associated with deficiencies in various lysosomal degradative enzymes and aberrant lysosomal accumulation of lipids (Blanchette-Mackie, 2000; Gieselmann, 1995; Pentchev et al., 1987; Platt et al., 1997). Cholesterol accumulation is closely related to the expression of MLBs in the Niemann-Pick lysosomal storage diseases.
Although Niemann-Pick A and B are associated with sphingomyelinase deficiency, Niemann-Pick C and D are caused by impaired intracellular cholesterol trafficking (Kolodny, 2000; Pagano et al., 2000). Patients with Niemann-Pick C disease are deficient for expression of the NPC1 protein implicated in the regulation of intracellular cholesterol traffic (Blanchette-Mackie, 2000; Ory, 2000; Pentchev et al., 1987). A class of drugs (class 2 amphiphiles such as U18666A) impairs cellular cholesterol traffic and results in the accumulation of cholesterol in late endosomes, lysosomes and MLBs mimicking Niemann-Pick C disease (Butler et al., 1992; Lange et al., 1998; Liscum et al., 1989).

Fibroblasts from patients with sphingolipid storage diseases show defects in lipid transport and sorting (Pagano et al., 2000). In many of these diseases, altered cholesterol homeostasis leads to perturbations in lipid traffic (Puri et al., 1999). Increased cellular cholesterol alters sphingolipid trafficking, resulting in its delivery not to the Golgi from the plasma membrane but rather to endolysosomal compartments such that altered trafficking of sphingolipids can be considered a diagnostic tool for the identification of sphingolipid storage diseases (Chen et al., 1999). U18666A-mediated cholesterol accumulation can be reduced by overexpression of Rab 7 and 9 GTPases suggesting that the modulation of endosomal lipid composition can impact on the delivery of material to lysosomes (Choudhury et al., 2002; Kobayashi et al., 1999; Pagano et al., 2000). Cholesterol accumulation in lysosomal storage diseases has further been proposed to result in the trapping of lipid raft components in late endocytic structures that could result in the formation of MLBs (Lusa et al., 2001; Simons and Grueenberg, 2000).

Transfection of the Mv1Lu mink lung alveolar type II cell line, which does not express MLBs, with β1-6-N-acetyl-glucosaminyl-transferase V (Mgat5) results in the stable expression of cytoplasmic MLBs implicating glycosylation of lysosomal glycoproteins in MLB expression (Hariri et al., 2000). Interestingly, defects in galactosidases (galactosidosis) and sialidases (sialidosis) as well as accumulation of polylactosamine are associated with MLB accumulation in lysosomal storage diseases (Allegrezna et al., 1989; Amano et al., 1983; Berra et al., 1986; DeGasperi et al., 1990). L-PHA labelling of Mgat5 generated β1-6 GlcNAc-branched N-glycans in MLBs has also been described in melanomas and other cancers (Handerson and Pawelek, 2003).

Multilamellar bodies are therefore ubiquitous organelles expressed under various physiological and pathological conditions. The similar morphology and lysosomal nature of these organelles in various cell types argues that common mechanisms must necessarily regulate their formation. Studies of MLB biogenesis in the Mgat5-transfected type II alveolar Mv1Lu cells showed that MLB formation could be prevented by treatment with the protease inhibitor, leupeptin, resulting in the accumulation of dense autophagic vacuoles, and that inhibition of autophagy with 3-methyladenine (3-MA) also blocked MLB expression (Hariri et al., 2000). Similarly, lysosomal protein degradation is required for MLB formation in primary human lung type II alveolar cells (Guttentag et al., 2003). Early electron microscopic studies of fetal lung describing the coordinated loss of glycogen with the accumulation of multilamellar bodies (Campiche et al., 1963; O’Hare and Sheridan, 1970) and the presence of cytoplasmic glycogen in lamellar bodies in type II epithelial cells in fetal lung, further support a role for autophagy in MLB formation (Stahlman et al., 2000; Weaver et al., 2002). However, despite the known ability of cholesterol accumulation to stimulate MLB formation in lysosomal storage disease models, the impact of lysosomal cholesterol on the biogenetic mechanisms underlying MLB formation remains undetermined.

MLBs in Mgat5-transfected Mv1Lu cells are rich in phospholipids and low in cholesterol and we have used the NPC-mimicking amphiphilic drug U18666A [3β-(2-diethylaminoethoxy)-androstenone] to study the role of lysosomal cholesterol accumulation on the biogenesis of MLBs in the Mv1Lu type II alveolar cell line. U18666A stimulates MLB formation in both parental and Mgat5-transfected Mv1Lu cells. It further transforms the dense autophagic vacuoles that accumulate following extended treatment with the lysosomal protease inhibitor leupeptin into MLBs. Cholesterol therefore induces MLB formation in Mv1Lu type II alveolar cells independently of both Mgat5 expression and lysosomal degradation. Although inhibition of autophagy with the PI 3-kinase inhibitor 3-methyladenine prevents the expression of concentric lamella in the U18666A-induced cholesterol-rich lysosomal vacuoles, stimulation of autophagy by serum starvation results in an increased size of MLBs presenting the concentric lamellar morphology typical of MLBs. Phospholipid-rich MLBs as well as U18666A-induced cholesterol-rich MLBs are accessible to fluid-phase uptake after 4 hours and labelled with Lysotracker Red, identifying them as late lysosomal organelles or autolysosomes. Similarly, the non-lamellar vacuoles induced in the presence of 3-MA are late lysosomal organelles but exhibit significantly reduced labelling for the autolysosomal marker monodansylcadaverine. Multiple factors, including cholesterol content, glycosylation and autophagy, can therefore contribute to the formation of concentric lamellae within secondary lysosomes and autolysosomes.

**Materials and Methods**

**Chemicals**

Filipin complex, leupeptin, 3-MA, MDC and gelvatol were purchased from Sigma (St Louis, MO) and Alexa 488 goat anti-mouse IgG, Alexa 568 goat anti-mouse IgG, Alexa 647 goat anti-mouse IgG secondary antibodies, Lysotracker Red, Sytox Green and FITC-dextran (molecular weight 10,000; lysine-fixable) were from Molecular Probes (Eugene, OR). U18666A was purchased from Biomol (Plymouth Meeting, PA), G418 from Invitrogen (Burlington, ON) and Nile Red from ICN Biomedicals (Costa Mesa, CA).

**Cell culture**

Normal Mv1Lu mink lung epithelial cells, the M9 clone of Mv1Lu cells transfected with Mgat5 (Demetriou et al., 1995) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with glutamine, vitamins, non-essential amino acids (Invitrogen, Burlington, ON) and 10% FBS (Medicorp, Laval, QC) in an air/5% CO2 atmosphere at constant humidity at 37°C. To maintain the phenotype of Mgat5-transfected Mv1Lu cells, the medium was supplemented with G418 at a final concentration of 600 µg/ml (Hariri et al., 2000). Cells were plated at a density of 40,000 cells/cm² and the medium was replaced every 2 days. To induce formation of cholesterol-rich MLBs, medium was supplemented with U18666A (1:10,000 of a 10 mg/ml stock solution in ethanol) for 24 hours.
Leupeptin was added to the medium at a concentration of 2 μg/ml. 3-
MA, at a final concentration of 10 mM, was dissolved directly in the
medium, which was then sterilized by filtering (Hariri et al., 2000).

Immunofluorescence
Normal and Mgt5-transfected Mv1Lu cells were grown on glass
coverslips for 6 days and then processed for immunofluorescence as
previously described (Hariri et al., 2000). Cells were fixed with 3% paraformaldehyde for 15 minutes, washed with PBS supplemented
with 0.1 mM Ca²⁺ and 1 mM Mg²⁺ (PBS-CM) and then incubated for
15 minutes with PBS-CM supplemented with 0.2% BSA to reduce
non-specific binding and 0.07% saponin to permeabilize cellular
membranes. AC17 anti-LAMP-2 antibody (Nabi et al., 1991; Nabi
and Rodriguez-Boulan, 1993) was used to determine the cellular
distribution of LAMP-2 using Alexa 488 goat anti-mouse IgG as
secondary antibody. Intracellular cholesterol was visualized by filipin
labelling (1:25 of a stock solution of 10 mg/ml in DMSO). Phospholipid content was determined by mounting coverslips in
gelvatol containing Nile Red (1:1000 of a saturated solution).

To follow fluid-phase endocytosis, cells were incubated for various
periods of time with lysine-fixable FITC-Dextran (5 mg/ml) in cell
media. Cells were then washed with PBS-CM and processed for
immunofluorescence as described above. Visualisation of acidic
organelles was performed by incubation of cells for 10 minutes with
0.1 μM Lysotracker Red added to the medium before prior fixation and
labelling with filipin. Labelled cells were viewed with a 100×
Neofluor objective of a Zeiss Axioskop fluorescent microscope
equipped with UV, FITC and rhodamine filter sets, a QImaging Retiga
CCD camera and Northern Eclipse imaging software (Empix
equipped with UV, FITC and rhodamine filter sets, a QImaging Retiga
NeoFluor objective of a Zeiss Axiophot fluorescent microscope
/H11003
immunofluorescence as described above. Visualisation of acidic
media. Cells were then washed with PBS-CM and processed for
periods of time with lysine-fixable FITC-Dextran (5 mg/ml) in cell
media. Cells were then washed with PBS-CM and processed for
immunofluorescence as described above. Visualisation of acidic
organelles was performed by incubation of cells for 10 minutes with
0.1 μM Lysotracker Red added to the medium before prior fixation and
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CCD camera and Northern Eclipse imaging software (Empix
/H11003

Electron microscopy
Cells were rinsed with 0.1 mM sodium cacodylate, pH 7.3 and then
fixed for 1 hour with 2% glutaraldehyde at 4°C. After fixation, the
cells were rinsed with cacodylate buffer, scraped from the Petri dish,
pelleted and post-fixed with 2% osmium tetroxide at 4°C. The cells
were dehydrated and then embedded in LR-White resin. Ultra-thin
sections were prepared and treated with uranyl acetate and lead citrate
to enhance contrast. The sections were visualized with a Zeiss
CERM902 electron microscope. Quantification of the expression of
MLBs and of swollen lysosomal vacuoles in the 3-MA experiments
was determined by circumscribing the cytoplasm (excluding the
nucleus) and the MLBS and non-lamellar lysosomal vacuoles from six
images at 3000× magnification and determining the area of the
circumscribed regions. MLBS were defined as membrane-bound
cytoplasmic organelles presenting at least three distinct
circumferential concentric membrane lamellae. MLBS were
composed either completely of concentric lamellae or of concentric
lamellae surrounding a single dense core (Hariri et al., 2000). Swollen
lysosomal vacuoles were defined by the presence of multiple internal
structures surrounded by a limiting membrane and could be
morphologically distinguished from MLBS.

Results
MLB expression in type II alveolar cells following
U18666A-mediated lysosomal cholesterol accumulation
To assess the phospholipid and cholesterol distribution of the
MLBS expressed upon Mgt5 transfection of Mv1Lu cells,
both parental Mv1Lu and the M9 clone of Mgt5-transfected
Mv1Lu cells (Demetriou et al., 1995; Hariri et al., 2000)
were labelled for Nile Red, a phospholipid-specific dye that labels
MLBS of alveolar type II cells (Gonzales et al., 2001; Guttentag
et al., 2003), and for filipin, a cholesterol-specific dye.
Although neither Nile Red nor filipin labelling associated with
the LAMP-2-positive lysosomes of Mv1Lu cells (Fig. 1A-D),
the multiple large LAMP-2-positive lysosomal structures that

| Table 1. Quantification of MLB expression in Mv1Lu cells and M9 clones transfected with Mgt5 before and after treatment with U18666A and 3-MA |
|--------------------|----------|-----------|----------|----------|
| Cell type          | Multilamellar bodies (MLBs) | Non-lamellar lysosomal vacuoles |  |
|                    | Size range | % cell area | Size range | % cell area |
| Mv1Lu control      | 1 4.6      | 0.13       | –         | –          |
| Mv1Lu+U18666A      | 130 0.3-6.2 (1.6) 4.35±0.8 | –         | –         |
| Mv1Lu+3MA          | 0 0       | 0          | 62 0.5-3.5 (1.3) 1.1±0.5 |
| Mv1Lu+U18666A+3MA  | 7 0.2-5.4 (1.3) 0.02±0.05 | 86 0.8-6.0 (1.7) 3.5±0.7 |
| M9 control         | 107 0.1-6.7 (1.7) 5.1±0.6 | –         | –         |
| M9+U18666A         | 169 0.1-7.8 (1.9) 7.4±0.9 | –         | –         |
| M9+3MA             | 22 0.1-5.8 (1.2) 0.4±0.3 | 76 0.1-3.3 (1.1) 1.26±0.7 |
| M9+U18666A+3MA     | 9 0.1-6.1 (1.5) 0.4±0.3 | 100 0.1-7.4 (1.9) 6.5±0.5 |
correspond to MLBs in the M9 clone of Mga5-transfected Mv1Lu alveolar cells (Hariri et al., 2000) were labelled for Nile Red indicating that they are rich in phospholipids (Fig. 1E-H). Labelling of cholesterol with filipin shows that the lumen of the MLBs is not labelled although a peripheral filipin-positive ring is frequently observed to circumscribe the Nile Red positive MLBs (Fig. 1G,Q-T), consistent with the fact that in type II alveolar cells, cholesterol is mainly localized to the limiting membrane of the organelles (Orgeig and Daniels, 2001; Punnonen et al., 1988).

Treatment of both Mv1Lu and Mga5-transfected M9 cells with U18666A resulted in the expression of large LAMP-2-positive vacuoles labelled for both Nile Red and filipin, reflecting the ability of U18666A to induce cholesterol accumulation in both lysosomes and MLBs, respectively (Fig. 1I-P). MLBs are not observed in Mv1Lu cells using electron microscopy, whereas M9 cells present multiple cytoplasmic MLBs (Fig. 2A,B and Table 1), as previously reported (Hariri et al., 2000). In both cell lines, numerous MLBs are observed upon treatment with U18666A (Fig. 2C,D and Table 1) indicating that lysosomal accumulation of cholesterol results in MLB formation independently of Mga5 expression levels. In Mv1Lu cells, lysosomal cholesterol accumulation due to U18666A treatment therefore results in de novo formation of MLBs. In M9 cells, U18666A treatment increases the number of MLBs and the cytoplasmic area covered by MLBs (Table 1) indicating that cholesterol accumulation occurs within pre-existing phospholipid-rich MLBs without disrupting their lamellar morphology but also induces new MLBs.

Cholesterol accumulation overrides the role of lysosomal degradation but not autophagy in MLB formation

MLBs are lysosomal organelles and express various lysosomal hydrolases (de Vries et al., 1985; DiAugustine, 1974; Hatasa and Nakamura, 1965; Hook and Gilmore, 1982). The previously demonstrated ability of leupeptin treatment to prevent MLB formation in Mga5-transfected Mv1Lu cells (Hariri et al., 2000) led us to determine whether U18666A could induce MLB formation in leupeptin-treated M9 cells. After 96 hours in the presence of leupeptin, Mv1Lu and M9 cells express large LAMP-2/Nile Red-positive vacuoles and treatment with U18666A for 24 hours results in the formation of vacuoles strongly labelled for filipin, essentially identical to that observed in cells not treated with leupeptin (data not shown). Treatment of Mv1Lu cells with leupeptin for 4 days induced the accumulation of autolysosomes as viewed by electron microscopy (Fig. 3A). In M9 cells, leupeptin treatment resulted in the disappearance of MLBs and their replacement by large autolysosomes (Fig. 3B), as previously reported (Hariri et al., 2000). Treatment of leupeptin-treated cells with U18666A for the final 24 hours resulted in the induction of MLBs and
disappearance of autolysosomes in both cell types (Fig. 3C,D). U18666A-mediated accumulation of cholesterol therefore results in the formation of concentric lamellar structures within autolysosomes independently of protein degradation.

Addition of 3-MA inhibits autophagy at the sequestration step (Seglen and Gordon, 1982) and was previously shown to prevent MLB formation in Mgat5-transfected Mv1Lu cells (Hariri et al., 2000). M9 cells incubated with 3-MA for 3 days no longer expressed large LAMP-2/Nile Red-positive vacuoles and Nile Red labelling presented a diffuse cytoplasmic distribution (Fig. 4E-H), similar to that of untreated (Fig. 1) and 3-MA-treated Mv1Lu cells (Fig. 4A-D). Addition of U18666A to 3-MA-treated Mv1Lu and M9 cells for 24 hours induces the formation of large LAMP-2/Nile Red/filipin-positive vacuoles (Fig. 4I-P). As previously reported (Hariri et al., 2000), 3-MA treatment of both Mv1Lu- and Mgat5-transfected M9 cells results in the appearance of vacuoles that do not present internal concentric lamella and that are morphologically distinct from MLBs (Fig. 5A,D). U18666A treatment of 3-MA-treated Mv1Lu and M9 cells resulted in the expression of non-lamellar vacuoles morphologically similar to those induced by 3-MA in the absence of U18666A but larger in size (Fig. 5B,C,E,F and Table 1). In the presence of U18666A, the non-lamellar vacuoles represent the only cytoplasmic structures that could correspond to the filipin-labelled LAMP-2-positive vacuoles observed by immunofluorescence labelling (Table 1). The non-lamellar vacuoles observed in the presence of 3-MA therefore correspond to...
Fig. 4. U18666A treatment induces accumulation of swollen LAMP-2-positive vacuoles in the presence of the autophagy inhibitor 3-MA. Untransfected Mv1Lu (A-D,I-L) and M9 (E-H,M-P) cells were grown in medium supplemented with 10 mM 3-MA for 3 days and selected cultures were incubated with U18666A for the final 24 hours (I-P). The cells were fixed and then triple labelled with anti-LAMP-2 (A,E,I,M), Nile Red (B,F,J,N) and filipin (C,G,K,O). Merged images (D,H,L,P) show LAMP-2 in green, Nile Red in red and filipin in blue. Large phospholipid-rich, LAMP-2-positive vacuoles are not observed following 3-MA treatment but U18666A is still able to induce the formation of swollen, filipin-positive lysosomal vacuoles. Bar, 5 µm.
U18666A-induced cholesterol-rich lysosomal vacuoles. Treatment with 3-MA therefore prevents both the expression of MLBs in M9 cells and the ability of cholesterol accumulation to reorganize the internal membranes of lysosomal and autophagic vacuoles into the concentric, circumferential lamella typical of MLBs.

The MLBs of untreated and U18666A-treated M9 cells (Fig. 6A,C) are labelled for monodansylcadaverine (MDC), a marker for autophagic vacuoles (Biederbick et al., 1995; Munafò and Colombo, 2001). Following 3-MA treatment, the intensity of MDC labelling is significantly reduced in both untreated and U18666A-treated cells (Fig. 6B,D). The continued presence of large, swollen vacuoles labelled for Nile Red in U18666A, 3-MA-treated cells allowed us to quantify vacuole-associated MDC labelling. Untreated and U18666A-treated cells present a similar mean MDC intensity that is significantly reduced upon 3-MA treatment reflecting the ability of 3-MA to inhibit autophagy in this cell line (Fig. 6E) as previously described (Hariri et al., 2000). Serum starvation stimulates macroautophagy in various cell lines (Munafò and Colombo, 2001; Susan and Dunn, 2001). Starvation of both untransfected and Mga5-transfected Mv1Lu cells following U18666A treatment leads to an increase in the size of filipin-positive vacuoles after 3 and 6 hours as well as an increase in the total area covered by filipin-positives vacuoles per cell (Fig. 7). The vacuoles of serum-starved cells still present a multilamellar morphology (data not shown). Stimulation of macroautophagy is therefore associated with increased size and expression of MLBs.

3-MA does not affect fluid-phase endocytosis to lysosomes

The drug 3-MA is an inhibitor of PI 3-kinase (PI3K) (Blommaart et al., 1997) and the PI3K product phosphatidyl inositol 3-phosphate [PI(3)P] is known to be involved in endocytic trafficking as well as the autophagic process (Petiot et al., 2000; Simonsen et al., 2001; Wurmser et al., 1999). Inhibition of PI3K activity has also been shown to induce the redistribution of lysosomal glycoproteins from lysosomes to mannose-6-phosphate receptor-negative, acid hydrolase-negative late endosomal compartments (Reaves et al., 1996). We therefore examined whether 3-MA treatment was preventing endocytosis in Mga5-transfected Mv1Lu cells and whether the U18666A LAMP-2-positive vacuoles are late endocytic or lysosomal structures. Uptake of the fluid-phase marker FITC-dextran to phospholipid and cholesterol-rich MLBs was followed in untreated and 3-MA-treated cells. FITC-dextran is not present in LAMP-2-positive phospholipid- and cholesterol-rich MLBs of M9 cells after endocytosis for 30 minutes, but accumulates in these structures after 4 hours (Fig. 8). Similar results were observed for Mv1Lu and M9 cells treated with U18666A in the absence or presence of 3-MA (Fig. 8). Quantification revealed that delivery of FITC-dextran to LAMP-2/Nile Red-labelled lysosomal vacuoles was essentially identical irrespective of the presence of U18666A and/or 3-MA (Fig. 8S). Labelling of the majority of the vacuoles after 4 hours’ uptake identifies them as late lysosomal structures, consistent with the derivation of MLBs from autolysosomes (Hariri et al., 2000). Inhibition of autophagy in the presence of 3-MA (Hariri et al., 2000), suggests that U18666A is acting on secondary lysosomes of Mv1Lu and M9 cells. The equivalent rate of delivery of FITC-dextran to the MLBs of untreated M9

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Fig. 5. Swollen vacuoles induced by U18666A in the presence of 3-MA lack concentric lamella. Untransfected Mv1Lu (A–C) and Mga5-transfected M9 (D–F) cells were incubated with 10 mM 3-MA for 3 days and selected cultures were incubated with U18666A for 24 hours (B,C,E,F) prior to processing for electron microscopy. 3-MA treatment results in the disappearance of MLBs in M9 cells and expression of membrane-bound, non-lamellar inclusion bodies that lack concentric lamella. Bar, 0.5 µm (A,C,E,F); 2 µm (B,D).

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Fig. 6. Treatment with 3-MA reduces MDC labelling of swollen, lysosomal vacuoles. M9 cells were grown in regular medium (A,C) or in medium supplemented with 10 mM 3-MA for 3 days (B,D) and selected cultures were incubated with U18666A for the final 24 hours (C,D). Cells were incubated with MDC and Nile Red for 10 minutes and images of MDC labelling of unfixed cells are shown. (E) Mean MDC labelling intensity of lysosomal vacuoles positive for Nile Red was quantified in untreated M9 cells as well as in U18666A-treated M9 cells in the presence or absence of 3-MA (mean±s.d. of three independent experiments). MLBs present in untreated M9 cells and cells treated with U18666A are strongly labelled for MDC, however MDC labelling of swollen, lysosomal vacuoles formed in the presence of 3-MA treatment is significantly reduced. *P<0.005 when compared to intensity in control M9 cells. Bar, 10 µm.
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Fig. 7. Serum starvation stimulates of cholesterol-rich MLBs. (A) Untransfected Mv1Lu cells were grown for 6 days and U18666A was added for the final 24 hours. Selected cultures were then washed and incubated for 6 hours in serum-containing (B) or serum-free (C) media and labelled with filipin. The expression of filipin-labelled MLBs was quantified from both untransfected Mv1Lu (D) and M9 cells (E) incubated in serum-containing (empty bars) or serum-free (filled bars) media for 0.5, 1, 3 and 6 hours labelled with filipin and the nuclear dye Sytox Green. The average area of filipin-labelled vacuoles and the total area covered by these vacuoles per cell were quantified (means±s.e.m. of three independent experiments). *P<0.005, **P<0.01, ***P<0.05 compared to areas in control cells at the same time point. Bar, 10 μm.

Discussion
Multiple mechanisms modulate the expression of MLBs in the Mv1Lu type II alveolar cell line
MLB expression is usually lost upon long-term culture and in established cell lines of type II alveolar cells (Diglio and Kikkawa, 1977; Guttentag et al., 2003; Kawada et al., 1990; Rannels et al., 1987; Sannes, 1991; Tanswell et al., 1991). MLB expression in the mink Mv1Lu type II alveolar cell line can be induced by expression of β1-6GlcNAc-branched N-glycans (Hariri et al., 2000). MLBs were also induced in the Mv1Lu type II alveolar cell line by treatment with U18666A, a compound that promotes lysosomal cholesterol accumulation, independently of Mgat5 transgene expression. U18666A treatment has been shown to induce MLB formation in CHO cells (Lusa et al., 2001) but not in HeLa cells (Tomiyama et al., 2004), MDCK or mammary carcinoma cells (data not shown). Lysosomal cholesterol accumulation is therefore not sufficient to induce MLB formation and other cell-type specific factors are required. Nevertheless, the ability of both Mgat5 and cholesterol to independently promote MLB expression in the Mv1Lu type II alveolar cell line shows that multiple factors can modulate the expression of this organelle. The ability of cholesterol to override the need for lysosomal degradation in phospholipid-rich MLB formation demonstrates that the biogenesis of this organelle is a complex process that can follow different pathways. Varied lysosomal contents and biogenetic pathways are therefore permissive for MLB formation, consistent with the expression of these morphologically identical structures under normal physiological conditions in type II alveolar cells as well as pathologically in cancer and lysosomal storage diseases of different genetic origins.

Mgat5 and expression of β1-6GlcNAc-branched N-glycans products are upregulated with tumor progression (Dennis et al., 1999) and ‘coarse vesicles’ presenting a lamellar morphology are revealed by L-PHA, a probe for branched N-glycans, in various cancers (Handerson and Pawelek, 2003). The expression of β1-6GlcNAc-branched N-glycans on lysosomal glycoproteins may promote their expression or stability thereby contributing to lamella expression. Knockout of LAMP-2 results in the mistargeting of lysosomal hydrolases
Biogenesis of multilamellar bodies

An autophagic contribution promotes lamella formation in lysosomal organelles

Autophagy is involved in both sphingolipid and glycoprotein metabolism (Ghidoni et al., 1996) and, conversely, sphingolipid ceramide can modulate autophagy in HT-29 cells (Scarlatti et al., 2004). 3-MA and other inhibitors of PI3K activity block autophagy (Blommaart et al., 1997; Hariri et al., 2000; Munafò and Colombo, 2001; Seglen and Gordon, 1982). Our previous demonstration that 3-MA inhibits both autophagy and the formation of MLBs in Mgat5-transfected Mv1Lu cells (Hariri et al., 2000) was confirmed in this study by the disappearance of phospholipid-rich MLBs in 3-MA-treated cells (Figs 4, 5). Treatment with 3-MA did not prevent the accumulation of lysosomal cholesterol but the swollen, lysosomal structures no longer expressed the concentric lamellae typical of MLBs. The similar morphology of these swollen, lysosomal structures with
the smaller inclusion bodies seen upon 3-MA treatment in the absence of U18666A suggests that these two structures are similar lysosomal organelles that vary in size owing to accumulation of cholesterol. As seen in Table 1, 3-MA treatment of U18666A-treated cells resulted in essentially the complete transition from expression of MLBs to non-lamellar lysosomal vacuoles. The large size of U18666A-induced vacuoles enabled us to identify and compare the multilamellar and non-lamellar lysosomal structures in untreated and 3-MA-treated cells by light microscopy.

Inhibitors of PI3K also block vesicular transport from late endosomes to lysosomes (Punnonen et al., 1994) and impair both early endosome fusion and maturation of lysosomes (Mousavi et al., 2003; Reaves et al., 1996; Simonsen et al., 1998). More recently, PI3K signalling has been shown to more specifically regulate endosomal sorting (Petiot et al., 2003). The large non-lamellar LAMP-2-positive, cholesterol-rich vacuoles formed upon addition of U18666A to 3-MA are labelled by fluid-phase uptake of FITC-dextran after 4 hours but not 30 minutes and are positive for Lysotracker Red indicating that they are acidic, late lysosomal organelles. The non-lamellar vacuoles that accumulate in the presence of 3-MA and U18666A are therefore not pre-lysosomal compartments but correspond to late or secondary lysosomes. Furthermore, 3-MA does not affect endocytic accessibility of fluid-phase material to these late, lysosomal organelles. Biosynthetic trafficking of LAMP to lysosomes can include transit via early endosomes and/or the plasma membrane (Nabi et al., 1991; Peden et al., 2004) and recycling of LAMPS permits uptake of anti-LAMP antibodies to lysosomes (Lippincott-Schwartz and Fambrough, 1987; Saby et al., 1991; Williams and Fukuda, 1990). As observed for fluid-phase uptake of FITC-dextran, anti-LAMP-2 antibodies internalized by recycling LAMP-2 access MBs and non-lamellar vacuoles after 2-4 hours (data not shown).

Multivesicular bodies or endosomes have been observed to fuse with MBs in type II alveolar cells (Chevalier and Collet, 1972; Voorhout et al., 1993; Vorbroeker et al., 1995) and sorting of endosomal content to the internal vesicles of multivesicular bodies may represent a requisite aspect of MB formation (Piper and Luzio, 2001; Stahl and Barbieri, 2002; Weaver et
This study shows that autophagic delivery of phospholipids to late lysosomal organelles promotes MLB expression in the Mv1Lu type II alveolar cell line and, indeed, argues that MLBs are autolysosomes. However, autoradiographic studies have shown that phospholipids are delivered to MLBs of type II alveolar cells in intact lung directly from the Golgi apparatus (Chevalier and Collet, 1972). Furthermore, MLBs are absent from Mv1Lu cells, as in most type II alveolar-derived cells in culture (Diglio and Kikkawa, 1977; Guttentag et al., 2003; Kawada et al., 1990; Rannels et al., 1987; Sannes, 1991; Tanswell et al., 1991), and the ubiquitous expression of MLBs in various other cell types argues that multiple mechanisms may contribute to MLB expression. Indeed, we were able to induce MLB expression in Mv1Lu cells by modulating both protein glycosylation and lysosomal cholesterol content. Whether autophagy plays a critical or regulatory role in MLB formation and surfactant expression in lung type II alveolar cells remains to be determined. Nevertheless, regulation of the lipid composition of late lysosomal organelles by autophagy promotes the expression of MLBs and may modulate expression of these organelles under both physiological and pathological conditions.

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