

Biogenesis of Multilamellar Bodies via Autophagy

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Transfection of Mv1Lu mink lung type II alveolar cells with β 1–6-*N*-acetylglucosaminyl transferase V is associated with the expression of large lysosomal vacuoles, which are immunofluorescently labeled for the lysosomal glycoprotein lysosomal-associated membrane protein-2 and the β 1–6-branched *N*-glycan-specific lectin phaseolis vulgaris leucoagglutinin. By electron microscopy, the vacuoles present the morphology of multilamellar bodies (MLBs). Treatment of the cells with the lysosomal protease inhibitor leupeptin results in the progressive transformation of the MLBs into electron-dense autophagic vacuoles and eventual disappearance of MLBs after 4 d of treatment. Heterologous structures containing both membrane lamellae and peripheral electron-dense regions appear 15 h after leupeptin addition and are indicative of ongoing lysosome–MLB fusion. Leupeptin washout is associated with the formation after 24 and 48 h of single or multiple foci of lamellae within the autophagic vacuoles, which give rise to MLBs after 72 h. Treatment with 3-methyladenine, an inhibitor of autophagic sequestration, results in the significantly reduced expression of multilamellar bodies and the accumulation of inclusion bodies resembling nascent or immature autophagic vacuoles. Scrape-loaded cytoplasmic FITC-dextran is incorporated into lysosomal-associated membrane protein-2–positive MLBs, and this process is inhibited by 3-methyladenine, demonstrating that active autophagy is involved in MLB formation. Our results indicate that selective resistance to lysosomal degradation within the autophagic vacuole results in the formation of a microenvironment propitious for the formation of membrane lamella.

INTRODUCTION

Multilamellar bodies (MLBs) are membrane-bound cellular organelles, which vary in size from 100–2400 nm, are composed of concentric membrane layers, and frequently exhibit an electron-dense core. MLBs are found in numerous cell types where they function in lipid storage and secretion (Schmitz and Müller, 1991). In lung type II alveolar cells, MLBs function as secretory granules whose exocytosis results in the deposition of the tubular myelin forms of surfactant on the surface of the alveolae (Hatasa and Naka-

mura, 1965; Ryan *et al.*, 1975; Williams, 1977). The surfactant film over the alveolar epithelium regulates the surface tension at the air–cell interface and protects the alveola from collapse during respiration (Haagman and van Golde, 1991).

Although the secretory function of MLBs in type II alveolar cells is well established, the precise mechanism of MLB biogenesis remains unclear. Autoradiographic studies of murine type II alveolar cells of mouse lungs showed that although phospholipids labeled with [³H]choline are delivered directly from the Golgi to the MLB, proteins metabolically labeled with [³H]leucine are visualized within multivesicular bodies before delivery to MLBs (Chevalier and Collet, 1972). Surfactant proteins A, B, and C are delivered via multivesicular bodies to MLBs, and multivesicular bodies are proposed as the site of processing of surfactant precursor to mature forms; both multivesicular bodies and MLBs express the lysosomal marker CD63 and are therefore part of the lysosomal pathway (Voorhut *et al.*, 1992, 1993). The lysosomal nature of the MLB has been demonstrated by the localization of various lysosomal enzymes to this organelle (Balis and Conen, 1964; Hatasa and Nakamura, 1965; Goldfischer *et al.*, 1968; Hoffman, 1972; DiAugustine, 1974;

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Abbreviations used: AV_d, degradative autophagic vacuole; AV_i, nascent or immature autophagic vacuole; GlcNAc-TV, β 1–6-*N*-acetylglucosaminyl transferase V; LAMP-2, lysosomal-associated membrane protein-2; 3-MA, 3-methyladenine; L-PHA, phaseolis vulgaris leucoagglutinin; MLB, multilamellar body; PBS/CM, PBS supplemented with 0.1 mM Ca²⁺ and 1 mM Mg²⁺.

Heath *et al.*, 1976; Hook and Gilmore, 1982; de Vries *et al.*, 1985).

It has been previously suggested based on morphological criteria that MLBs form via cellular autophagy (Balis and Conen, 1964; Sorokin, 1967; Flaks and Flaks, 1972; Stratton, 1978). Autophagy is a normal degradative process that exists in all eukaryotic cells and is stimulated in response to a variety of environmental stresses, which necessitate the use of autophagic mechanisms to enable cellular survival (Seglen and Bohley, 1992; Dunn, 1994). Degradative autophagic vacuoles (AV_d) form after acquisition of lysosomal properties by nascent, immature autophagic vacuoles (AV_i), which present multiple limiting membranes and are considered to form by the sequestration of cytoplasm by smooth endoplasmic reticulum membranes (Dunn, 1990; Furuno *et al.*, 1990; Ueno *et al.*, 1991). The lysosomal nature of both the autophagic vacuole and the MLB supports a relationship between the two organelles; however, definitive evidence of a role for autophagy in MLB biogenesis has yet to be demonstrated.

Transfection of the immortalized Mv1Lu cell line, derived from mink lung type II alveolar cells, with β -1-6-*N*-acetylglucosaminyl transferase V (GlcNAc-TV), the enzyme responsible for the β -1-6 branching of *N*-glycans, which favors the addition of elongated polylactosamine chains, results in the loss of contact inhibition, decreased substrate adhesion, increased susceptibility to apoptosis and increased tumorigenicity in nude mice (Demetriou *et al.*, 1995). GlcNAc-TV-transfected Mv1Lu cells also exhibit increased phaseolus vulgaris leucoagglutinin (L-PHA) reactivity of lysosomal-associated membrane protein-2 (LAMP-2), demonstrating that increased GlcNAc-TV activity alters the β 1-6 branching of this heavily glycosylated lysosomal glycoprotein (Demetriou *et al.*, 1995). We show here that in contrast to untransfected Mv1Lu cells, which exhibit none or at best few MLBs, GlcNAc-TV-transfected Mv1Lu cells stably express numerous cytoplasmic MLBs. MLB formation in the GlcNAc-TV transfectants is reversibly regulated by leupeptin, an inhibitor of lysosomal proteases, demonstrating that lysosomal degradation is necessary for the formation of the membrane lamella of the MLB. It is also inhibited by 3-methyladenine (3-MA), a specific inhibitor of early stages in autophagic vacuole formation (Seglen and Gordon, 1982), and we demonstrate the necessary role for autophagy and autophagic vacuole biogenesis in MLB formation.

MATERIALS AND METHODS

Cell Culture

Mv1Lu mink lung epithelial cells, mock-transfected Mv1Lu cells (C1), and the GlcNAc-TV-transfected Mv1Lu cell lines (R2, M9, and M1) (Demetriou *et al.*, 1995) were grown in Dulbecco's modified Eagle's medium supplemented with glutamine, nonessential amino acids (Life Technologies, Oakville, Ontario, Canada), and 10% FBS (Immunocorp, Laval, Quebec, Canada) in an air-5% CO₂ atmosphere at constant humidity at 37°C. The medium of the transfected cell lines (C1, R2, M9, and M1) was supplemented with 600 μ g/ml G418 (Life Technologies) to maintain the transfected phenotype. For all experiments, cells were plated at a density of 40,000 cells/cm², and the medium was replaced every 2 d. Leupeptin (Roche Diagnostics, Laval, Quebec, Canada) was added to cell cultures at a concentration of 2 μ g/ml, and 3-MA (Sigma, St. Louis, MO) was added at a concentration of 10 mM.

Immunofluorescence

Cells cultured on glass coverslips were fixed by the addition of precooled (−80°C) methanol/acetone (80:20% vol/vol) directly to the coverslips and then placed at −20°C for 15 min. After fixation, the cells were rinsed extensively with PBS, pH 7.4, supplemented with 0.1 mM Ca²⁺ and 1 mM Mg²⁺ (PBS/CM), and then incubated for 15 min with PBS/CM containing 0.5% BSA at room temperature to reduce nonspecific binding. LAMP-2 distribution was determined using the AC17 anti-LAMP-2 antibody (Nabi *et al.*, 1991; Nabi and Rodriguez-Boulan, 1993) followed by FITC- or Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Detection of the distribution of L-PHA reactivity was performed using rhodamine-conjugated L-PHA (E-Y Laboratories, San Mateo, CA). After labeling, the coverslips were mounted in Airvol (Air Products and Chemicals, Allentown, PA). Labeled cells were viewed in a Zeiss (Thornwood, NY) Axioskop fluorescent microscope equipped with a 63× Plan Aplan objective and fluorochrome-selective filters. Images were photographed using Eastman Kodak (Rochester, NY) T-Max 400 film. Confocal microscopy was performed with the 60× Nikon (Tokyo, Japan) Plan Aplan objective of a dual-channel Bio-Rad (Hercules, CA) MRC 600 laser scanning confocal microscope equipped with a krypton/argon laser and printed using a Polaroid (Cambridge, MA) TX1500 video printer.

Electron Microscopy

Cells grown on Petri dishes were rinsed with 0.1 M sodium cacodylate, pH 7.3, and fixed with 2% glutaraldehyde for 60 min at 4°C. The fixed cells were rinsed in cacodylate buffer, scraped from the Petri dish, and collected by centrifugation. The cell pellet was post-fixed for 60 min with 2% osmium tetroxide at 4°C, dehydrated, and embedded in LR-White resin (MecaLab, Montreal, Quebec, Canada). Ultra-thin sections (80 nm) were contrasted with uranyl acetate and lead citrate and visualized with a Phillips (Eindhoven, The Netherlands) 300 or Zeiss CEM902 electron microscope. Quantification of the expression of MLBs and of inclusion bodies in the 3-MA experiments was determined by circumscribing the cytoplasm (excluding the nucleus), the MLBs, and the inclusion bodies from 10 images at 4400× magnification and determining the area of the circumscribed regions. MLBs were defined as membrane-bound cytoplasmic organelles that present at least three distinct circumferential concentric membrane lamellae. MLBs were composed either completely of concentric lamella or of concentric lamella surrounding a single dense core. Inclusion bodies, or AV_i, were defined by the presence of multiple internal structures surrounded by a limiting membrane composed of single or multiple membranes and could be morphologically distinguished from MLBs.

Scrape Loading of FITC-Dextran

FITC-dextran was scrape loaded into M9 cells essentially as previously described (McNeil *et al.*, 1984). Cells were plated overnight to semiconfluence and then rinsed three times in cold PBS/CM and incubated on ice for 15 min to chill the cultures. Cold PBS/CM (0.5 ml) containing 2.5 mg/ml lysine-fixable 10,000 molecular weight FITC-dextran (Molecular Probes, Eugene, OR) was added to the culture dish, and the cells were immediately scraped from the dish in the concentrated FITC-dextran solution. The cell suspension was rapidly diluted in 40 ml of cold PBS/CM and centrifuged in the cold to pellet the cells. The scrape loading was performed at 4°C, and the cells were rapidly diluted in cold PBS/CM to reduce the possibility of FITC-dextran uptake by fluid phase endocytosis. The cell pellet was resuspended in culture medium, and the cells were plated for 2, 24, 48, or 72 h in regular medium or in medium containing 10 mM 3-MA before fixation with 3% paraformaldehyde and immunofluorescence labeling with anti-LAMP-2 and Texas Red-conjugated secondary antibodies. Quantification of autophagic activity was per-

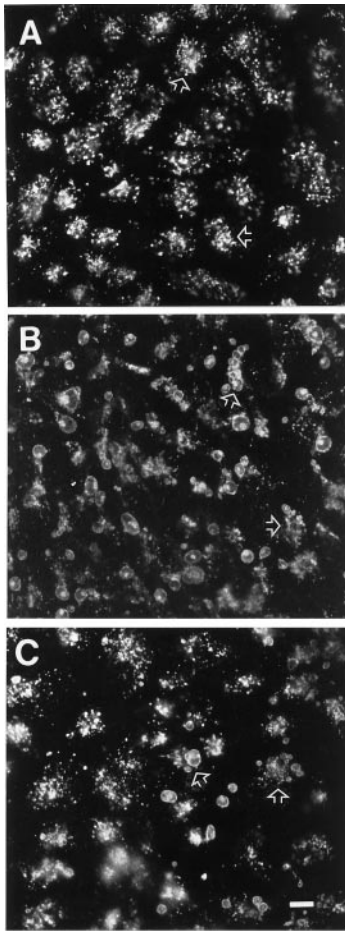


Figure 1. Expression of large lysosomal vacuoles in GlcNAc-TV-transfected Mv1Lu cells. Untransfected Mv1Lu cells (A) or GlcNAc-TV-transfected clones M9 (B) and M1 (C) plated for 6 d were immunofluorescently labeled with LAMP-2 to reveal the distribution of lysosomes in these cells. Clusters of lysosomes representing the lysosomes of individual cells are indicated by the arrows. Distinct swollen LAMP-2-positive lysosomal vacuoles are present in M9 and M1 cells but not in untransfected Mv1Lu cells. Bar, 10 μm .

formed by counting the number of FITC-dextran-loaded cells exhibiting FITC labeling of LAMP-2-positive lysosomal structures.

RESULTS

Increased Expression of MLBs in GlcNAc-TV-transfected Mv1Lu Cells

GlcNAc-TV transfection of Mv1Lu cells resulted in the obtention of clones M9 and M1, which exhibited significantly higher expression levels of GlcNAc-TV activity and increased L-PHA reactivity of LAMP-2 (Demetriou *et al.*, 1995). To assess the distribution of LAMP-2 and lysosomes in these cells, 6-d confluent cultures of untransfected Mv1Lu cells and M9 and M1 GlcNAc-TV transfectants were immunofluorescently labeled with antibodies to LAMP-2 (Figure 1). Clusters of LAMP-2-labeled lysosomes, as indicated by the

arrows, correspond to the perinuclear concentration of lysosomal organelles in an individual cell. In contrast to the punctate distribution of the lysosomal marker in Mv1Lu cells, anti-LAMP-2 antibodies label large vacuolar structures in both the M9 and M1 cell lines (Figure 1, B and C). To determine whether the increased β 1–6-branched *N*-glycans of the GlcNAc-TV transfectants are indeed localized to the large LAMP-2-positive lysosomal vacuoles present in the M9 and M1 transfectants, Mv1Lu cells and M9 cells were double immunofluorescently labeled for LAMP-2 and L-PHA, a lectin specific for the β 1–6 branching of polyacetylamines chains. By confocal microscopy, LAMP-2 and L-PHA colocalize in both the punctate LAMP-2 lysosomal labeling of Mv1Lu cells as well as the swollen LAMP-2-labeled vacuoles of M9 cells (Figure 2).

Electron microscopy of the different cell types revealed the presence of large MLBs in the M9 and M1 cell lines but not in the untransfected Mv1Lu cells (Figure 3). MLBs of M1 cells frequently exhibit a dense core surrounded by lamellae, whereas those of M9 cells exhibited a more uniform lamellar morphology and are larger. Expression of MLBs in untransfected Mv1Lu cells, mock-transfected C1 cells, and the GlcNAc-TV transfectants R2, M9, and M1 exhibiting increasing levels of GlcNAc-TV activity (Demetriou *et al.*, 1995; Table 1) was quantified by determining the extent of cytoplasmic area that was filled by MLBs in the different cells. Both the number of MLBs and the proportion of cytoplasmic area that they cover are significantly greater in high GlcNAc-TV-expressing M9 and M1 cells compared with untransfected Mv1Lu, mock-transfected C1, or low-expressing R2 cells (Table 1). Curiously, the area covered by MLBs is greater in M9 cells than in M1 cells, which express higher GlcNAc-TV levels; the number of MLBs in M1 cells is larger than in M9 cells, indicating that the difference between the two cell types is due to an increased size of MLBs in the M9 cells. MLBs $>2 \mu\text{m}^2$ in area are not observed in untransfected Mv1Lu, mock-transfected C1, or low-GlcNAc-TV-expressing R2 cells. Untransfected Mv1Lu cells can express MLBs, albeit very few, suggesting that these cells may have partially retained a differentiated type II phenotype. Mock-transfected C1 and low-GlcNAc-TV-expressing R2 cells (Demetriou *et al.*, 1995) exhibit more MLBs than untransfected Mv1Lu cells. However, the difference between the MLB/cytoplasm ratio of C1 and R2 cells and Mv1Lu cells is not statistically significant ($0.05 < p < 0.1$) and suggests that MLB expression in C1 and R2 cells may be due to subtle changes in the phenotype of the cells after transfection.

Lysosomal Degradation Is Necessary for MLB Biogenesis

The only cytoplasmic structures visualized in M9 or M1 cells by electron microscopy large enough to correspond to the large LAMP-2- and L-PHA-positive vacuoles identified in these cells by immunofluorescence labeling are MLBs. Furthermore, the expression of LAMP-2 in the MLBs of GlcNAc-TV-transfected Mv1Lu cells is consistent with the previously described lysosomal nature of this organelle (Balis and Conen, 1964; Hatasa and Nakamura, 1965; Goldfischer *et al.*, 1968; Hoffman, 1972; DiAugustine, 1974; Heath *et al.*, 1976; Hook and Gilmore, 1982; de Vries *et al.*, 1985; Voorhut *et al.*, 1992). To assess the role of lysosomal degradation on MLB expression, M1 cells were treated with the lysosomal

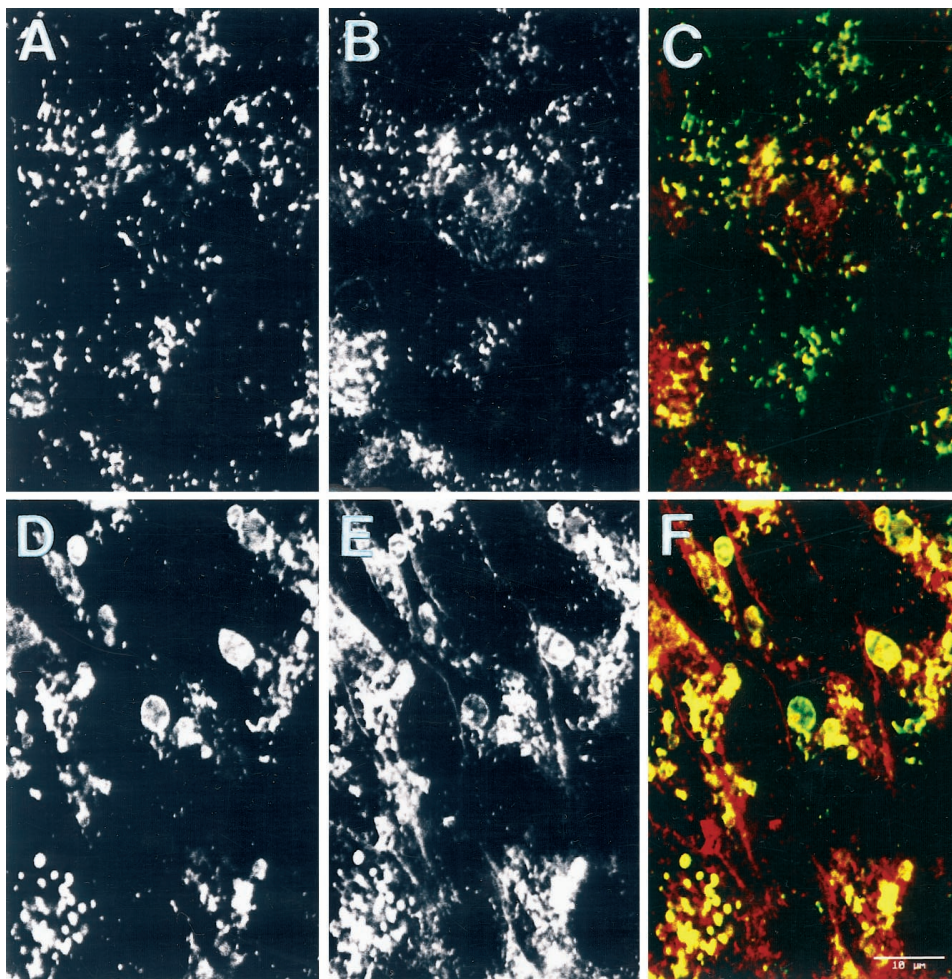


Figure 2. The swollen lysosomal vacuoles of GlcNAc-TV transfectants are labeled with L-PHA. Untransfected Mv1Lu (A–C) or GlcNAc-TV-transfected M9 (D–F) cells plated for 6 d were double immunofluorescently labeled with anti-LAMP-2 followed by FITC-conjugated anti-mouse secondary antibody (A and D) or with rhodamine-conjugated L-PHA (B and E). Merged images are presented in C and F (LAMP-2 in green, L-PHA in red). LAMP-2- and L-PHA-reactive β 1–6-branched oligosaccharides are localized to lysosomes of Mv1Lu cells as well as to the large lysosomal vacuoles of GlcNAc-TV M9 transfectants. Bar, 10 μ m.

protease inhibitor leupeptin (Figure 4). After 15 h of leupeptin treatment, MLBs exhibit electron-dense material around the periphery of the vacuole caused by the apparent fusion of MLBs with other lysosomal organelles and transfer of nonlamellar electron-dense material. In addition to these heterogeneous structures, smaller dense vacuoles accumulate. With increasing time in leupeptin-containing media, MLBs are no longer evident, and after 4 d of incubation with leupeptin, only large dense vacuoles are present in M1 cells (Figures 4E and 5A). Leupeptin treatment has been previously shown to induce the accumulation of AV_d (Furuno *et al.*, 1982; Ueno *et al.*, 1991; Yokota *et al.*, 1995), and the leupeptin-induced vacuoles in M1 cells are morphologically equivalent to AV_d (Figures 4E and 5A).

To determine whether removal of leupeptin and activation of lysosomal degradation could reverse this process and lead to the formation of MLBs, M1 cells treated with leupeptin for 4 d were then incubated in the absence of leupeptin and analyzed by electron microscopy. Fifteen hours after leupeptin washout, dense AV_d are still present in M1 cells; however, they appear to have lost some internal structure (Figure 5B). After 24 h the morphological transformation of the vacuoles commences, and numerous dense core bodies are present with some exhibiting internal lamellae (Figure

5C). Forty-eight hours after leupeptin removal, intermediates in the transformation of AV_d into MLBs can be visualized (Figure 5, D and E). The formation of single or multiple dense core lamellar structures within individual autophagic vacuoles can be clearly visualized. After 72 h in the absence of leupeptin, the cells exhibit MLBs similar to untreated cells (Figure 5F). The reversible regulation of MLB expression by leupeptin in GlcNAc-TV-transfected M1 cells demonstrates that lysosome fusion with MLBs and subsequent degradation of MLB contents by lysosomal hydrolases regulate lamella formation.

Role of Autophagy in MLB Biogenesis

Leupeptin is a general inhibitor of lysosomal protease activity, and although it blocks degradation of AV_d (Furuno *et al.*, 1982; Kovacs *et al.*, 1982; Ueno *et al.*, 1991; Yokota *et al.*, 1995), it is not a specific inhibitor of autophagy. To determine whether autophagy is specifically involved in MLB formation, we used 3-MA, which has previously been demonstrated to block autophagy at the initial sequestration step (Seglen and Gordon, 1982). By immunofluorescence, 3-MA treatment results in the disappearance of large LAMP-2-positive vacuoles in M9 cells (Figure 6). The LAMP-2-posi-

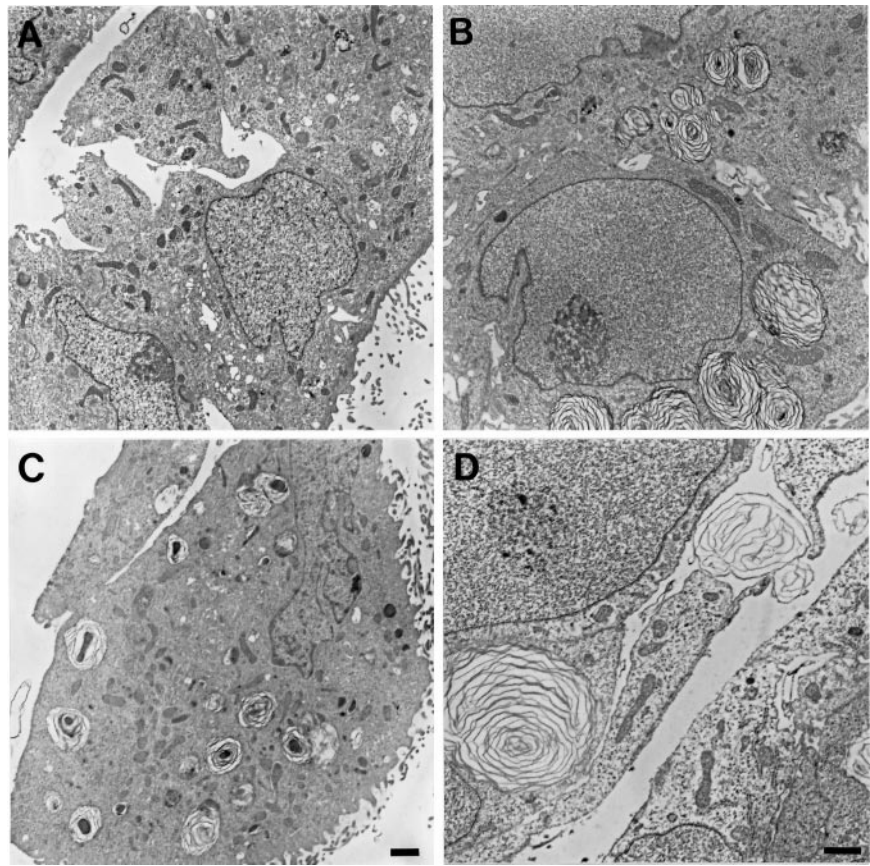


Figure 3. GlcNAc-TV transfectants express MLBs. Untransfected Mv1Lu cells (A) or GlcNAc-TV-transfected clone M9 (B and D) and M1 (C) cells plated for 6 d were processed for electron microscopy. Distinct MLBs are present in the cytoplasm of both M9 (B) and M1 (C) cells but are absent from untransfected Mv1Lu cells (A). The lamellar morphology of large MLBs is particularly evident in M9 cells (D). Bars, 1 μM (A–C); 0.5 μM (D).

tive lysosomes in both Mv1Lu and M9 cells treated with 3-MA (Figure 6, B and D) are slightly swollen compared with those of untreated Mv1Lu cells (Figure 6A); however, the large LAMP-2-positive vacuoles corresponding to MLBs in M9 cells (Figure 6C) are no longer visible in 3-MA-treated M9 cells (Figure 6D).

The ability of 3-MA to induce the disappearance of MLBs was confirmed by electron microscopy (Figure 7). In cells

treated with 3-MA, distinctive inclusion bodies accumulate, which do not present the circumferential membrane layers of MLBs (Figure 7, arrows). The limiting membrane of the inclusion bodies is formed of double or multiple membranes and surround multiple internal structures including multilamellar structures. These inclusion bodies can be morphologically distinguished from MLBs, whose concentric membrane layers surround only a single dense core. These

Table 1. Quantification of MLB expression in GlcNAc-TV-transfected Mv1Lu cells

Cell type	GlcNAc-TV (pmol/mg/h) ^a	Total no. of MLBs	Range of MLB size (μm^2)	Total cytoplasmic area (μm^2)	Total MLB area (μm^2)	Area MLB/ cytoplasm (%)
Mv1Lu	63 \pm 6	1	0.53–0.53	2022	0.53	0.03 \pm 0.03
C1	59 \pm 5	29	0.27–2.00	1875	22.5	1.1 \pm 0.6
R2	151 \pm 15	15	0.33–1.60	1882	12.3	0.6 \pm 0.3
M9	567 \pm 265	78	0.23–16.3	1690	223	13.0 \pm 1.1
M1	1082 \pm 308	98	0.22–14.0	1821	109	6.1 \pm 1.6

From 10 electron microscopy images (4400 \times) of each cell line, the area covered by MLBs and by cytoplasm (excluding the nucleus) in the complete image was determined. Using a two-tailed Student's *t* test, the ratio of MLB to cytoplasmic area in M9 cells is significantly different from both Mv1Lu and C1 cells ($p < 10^{-5}$). For M1 cells, the ratio of MLB to cytoplasmic area is also significantly different from both Mv1Lu ($p < 0.005$) and C1 ($p < 0.01$) cells. The significance of the difference in MLB/cytoplasm area between either C1 or R2 cells and Mv1Lu cells is of the order of $0.05 < p < 0.1$.

^a The values for GlcNAc-TV expression were taken from Dimetriou *et al.* (1995).

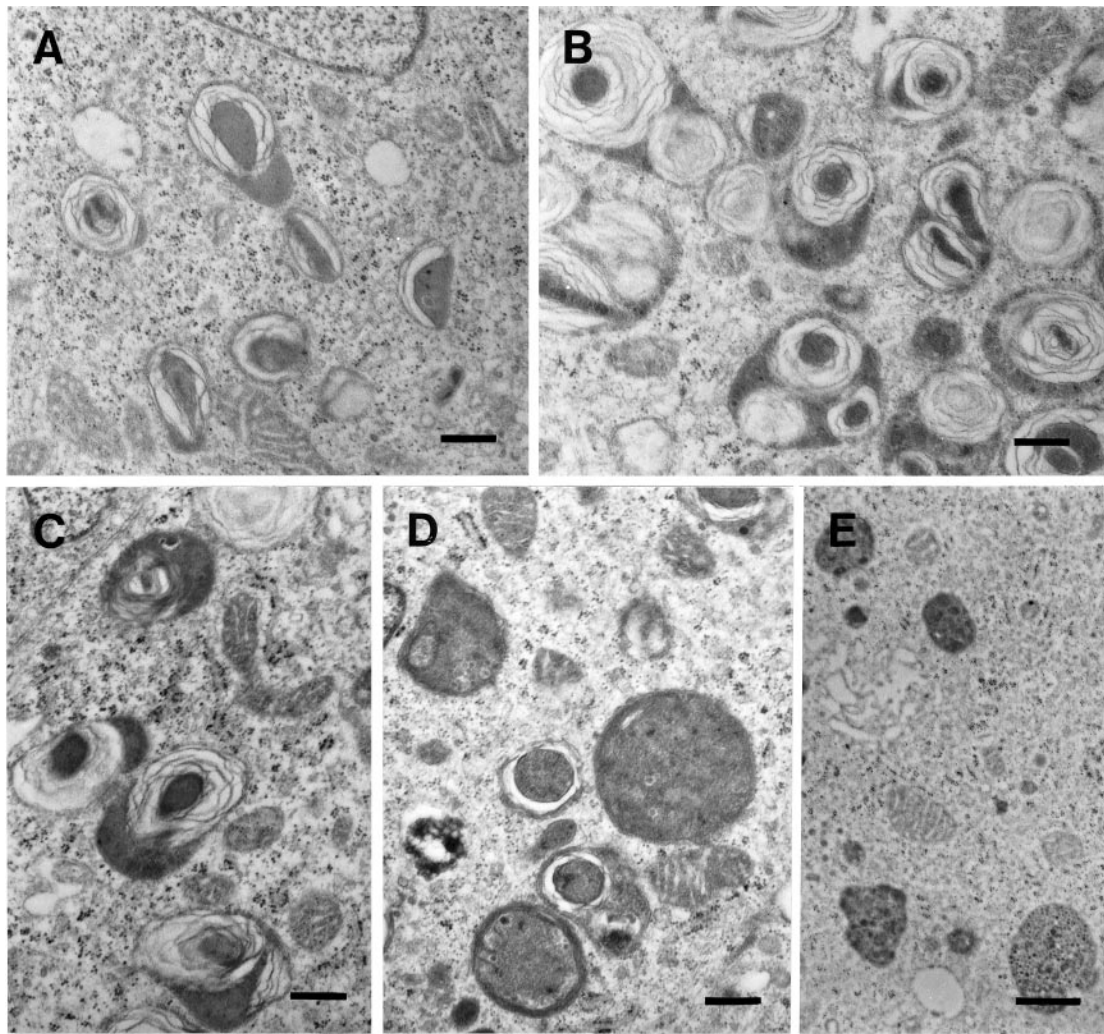


Figure 4. Gradual transformation of MLBs into AV_d by leupeptin treatment. GlcNAc-TV-transfected M1 cells were plated for 2 d in regular medium and then incubated for 15 (A and B), 48 (C), 72 (D), or 96 (E) h in the presence of 2 $\mu\text{g}/\text{ml}$ leupeptin and processed for electron microscopy. At early times after addition of leupeptin, dense material can be observed at the periphery of MLBs (A and B), and at later times dense vacuoles proliferate such that after 96 h autophagic vacuoles predominate, and MLBs are no longer present. Bars, 0.5 μm .

inclusion bodies are morphologically equivalent to AV_i , and their expression after 3-MA treatment is consistent with the role of 3-MA as an inhibitor of autophagy. Quantitative analysis of the effect of 3-MA treatment on MLB expression in M9 and M1 cells demonstrates that cells cultured in 10 mM 3-MA for 3 d exhibit significantly decreased expression of MLBs (Table 2). In some experiments, we observed the complete disappearance of MLBs. Relative to MLB expression in control cells, expression of inclusion bodies in 3-MA-treated cells is significantly reduced in all experiments. The ability of 3-MA to inhibit MLB formation identifies a role for autophagic vacuole maturation in MLB formation.

To demonstrate that 3-MA indeed blocks autophagy in GlcNAc-TV-transfected Mv1Lu cells and to confirm the role of autophagy in MLB biogenesis, M9 cells were scrape loaded with FITC-dextran. The procedure was performed at 4°C to minimize endocytic capture of FITC-dextran such

that the fluorescent marker was incorporated only into the cytoplasm of the cell. The scrape-loaded cells were washed in the cold to eliminate any free FITC-dextran and then fixed at various times after plating in regular or 3-MA-supplemented medium. At early times after plating in regular medium (2 h), the majority of cells exhibit a cytoplasmic distribution of FITC-dextran, which is excluded from vacuolar structures (Figure 8, A and B). After 48 h, the majority of cells plated in regular medium exhibit an accumulation of FITC-dextran in LAMP-2-positive vacuoles including the swollen structures equivalent to MLBs (Figure 8, C and D). However, if the cells are replated in 3-MA-containing medium after scraping, the FITC-dextran remains cytosolic, and no LAMP-2-positive vacuoles are labeled (Figure 8, E and F). The number of cells presenting FITC-dextran labeling in LAMP-2 positive vacuoles was counted from six experiments (Figure 9). With time an increasing number of

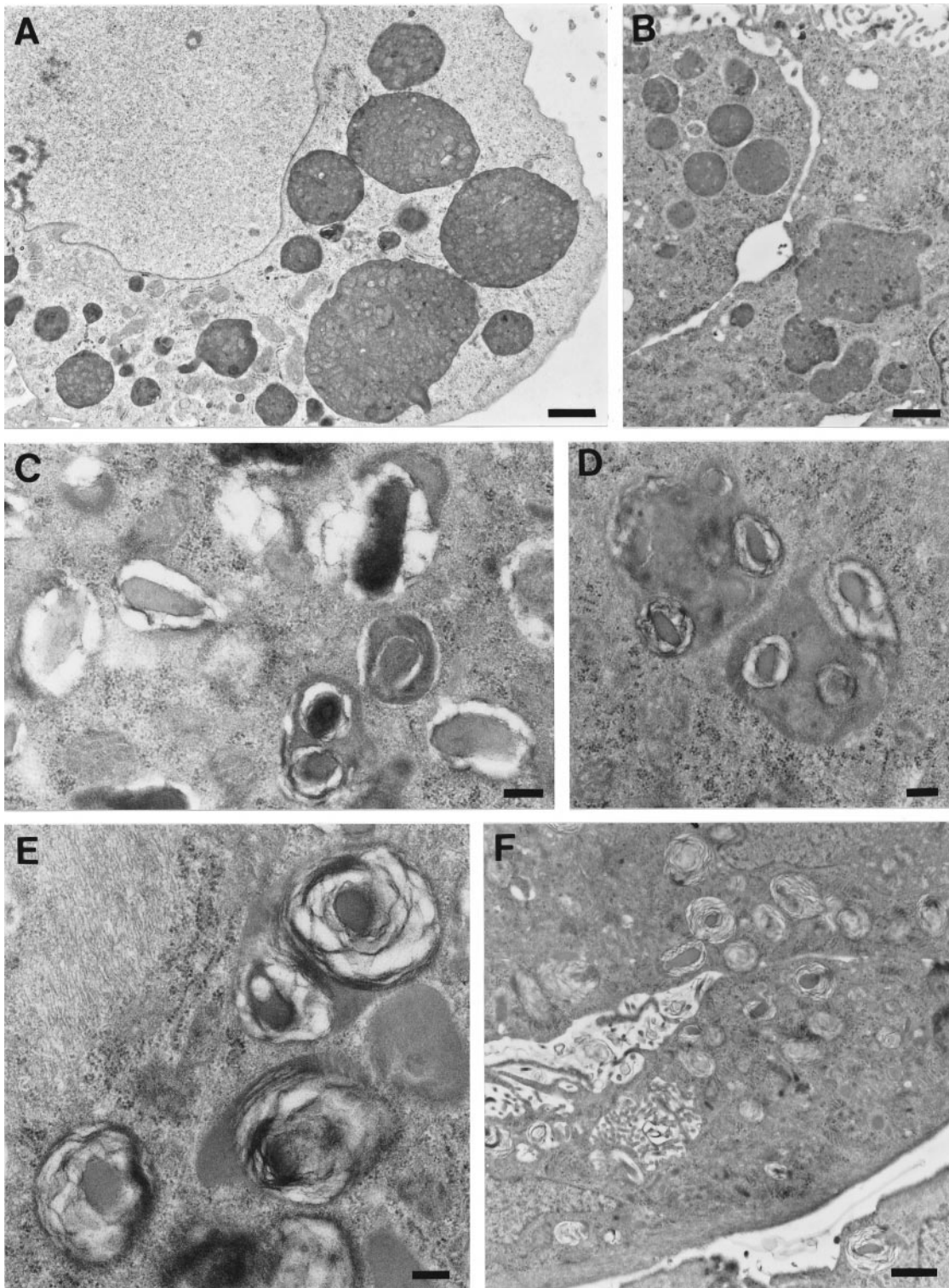


Figure 5. Formation of MLBs via autophagic vacuole degradation after leupeptin washout. GlcNAc-TV-transfected M1 cells were plated for 2 d and then incubated for 4 d in the presence of 2 $\mu\text{g}/\text{ml}$ leupeptin (A) and then washed and incubated in leupeptin-free medium for 15 (B), 24 (C), 48 (D and E), or 72 (F) h before being processed for electron microscopy. After leupeptin treatment, MLBs disappear, and large autophagic vacuoles are present (A). After 24 and 48 h, single or multiple foci of lamella form within the autophagic vacuole (C–E), and after 72 h the autophagic vacuoles transform into lamellar structures resembling those of untreated cells (F). Bars, 1 μm (A, B, and F); 0.2 μm (C–E).

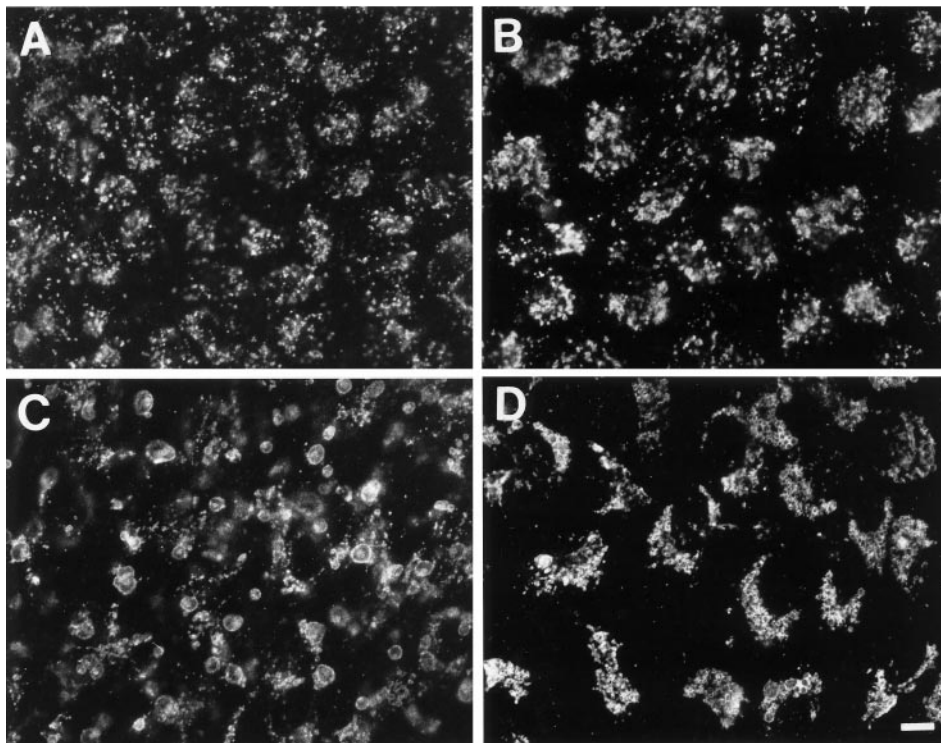


Figure 6. Inhibition of autophagy by 3-MA results in the disappearance of swollen LAMP-2-positive MLBs. Untransfected Mv1Lu (A and B) and GlcNAc-TV-transfected M9 (C and D) cells were incubated in regular medium (A and C) or medium supplemented with 10 mM 3-MA for 3 d (B and D) and then immunofluorescently labeled for LAMP-2. Although 3-MA treatment induces the formation of slightly swollen lysosomal structures in both Mv1Lu and M9 cells (B and D), it also results in the disappearance of the large LAMP-2-positive vacuoles, which correspond to MLBs in M9 cells (D). Bar, 10 μ m.

cells exhibit autophagic incorporation of FITC-dextran into LAMP-2-positive vacuoles. In the presence of 3-MA, essentially no cells exhibit a vacuolar labeling irrespective of the time of incubation in culture medium. The limited incorporation of scrape-loaded FITC-dextran into lysosomal vacuoles in the presence of 3-MA demonstrates that endocytic uptake of FITC-dextran by the cells was minimal and that 3-MA does indeed block autophagy in M9 cells. FITC-dextran transfer from the cytosol to large vacuoles of M9 cells, which correspond to MLBs, therefore demonstrates that active autophagy is involved in the formation of these organelles.

DISCUSSION

Expression of MLBs in GlcNAc-TV-transfected Mv1Lu Cells

β 1–6 branching of complex N-linked oligosaccharides is initiated by GlcNAc-TV and produces the preferred substrate for β -1-3-N-acetylglucosaminyl transferase (i), the rate-limiting enzyme implicated in poly lactosamine elongation (Holmes *et al.*, 1987; Yousefi *et al.*, 1991). Increased expression of poly lactosamine and the associated Lewis and blood group antigens are carcinoma markers (Fukuda, 1985; Hakamori, 1989). Modified expression of poly lactosamine is also associated with cellular differentiation of various cell types (Spillmann and Finne, 1987; Youakim *et al.*, 1989; Amos and Lotan, 1990; Lee *et al.*, 1990; Tuo *et al.*, 1992; Nabi and Rodriguez-Boulan, 1993). The decreasing poly lactosamine glycosylation of the lysosomal LAMP glycoproteins in cultured epithelial cells with time in culture is modulated in-

dependently of glycosyltransferase activities (Brockhausen *et al.*, 1991; Nabi and Dennis, 1998), and poly lactosamine glycosylation has been shown to be regulated by the Golgi residence time of the protein (Wang *et al.*, 1991; Nabi and Rodriguez-Boulan, 1993; Nabi and Dennis, 1998). Increased GlcNAc-TV expression is associated with increased poly lactosamine glycosylation in oncogenically transformed and undifferentiated cell lines (Yamashita *et al.*, 1985; Heffernan *et al.*, 1989; Yousefi *et al.*, 1991), indicating that GlcNAc-TV expression levels can regulate the expression of poly lactosamine oligosaccharides.

Transfection of the contact-inhibited lung epithelial cell line with GlcNAc-TV resulted in increased expression of L-PHA-reactive β 1–6-branched N-glycans and the expression of a partially transformed phenotype, including loss of the contact-inhibited phenotype, tumorigenicity in nude mice, and an increased propensity to apoptosis (Demetriou *et al.*, 1995). GlcNAc-TV expression in Mv1Lu cells is therefore associated with the expression of early events in cellular transformation. Mv1Lu cells are derived from lung type II alveolar cells, responsible for the elaboration of alveolar surfactant in the lung. Surfactant secretion by type II lung alveolar cells is mediated by MLBs whose presence in type II cells is a phenotypic characteristic of these cells (Haagman and van Golde, 1991). However, the differentiated type II alveolar phenotype is highly unstable in culture, and the expression of MLBs by primary type II cell cultures is maintained for only days after establishment of the cultures (Diglio and Kikkawa, 1977; Dobbs *et al.*, 1985). The ability of GlcNAc-TV transfection to induce the formation of MLBs in a cultured type II alveolar-derived cell line identifies a role

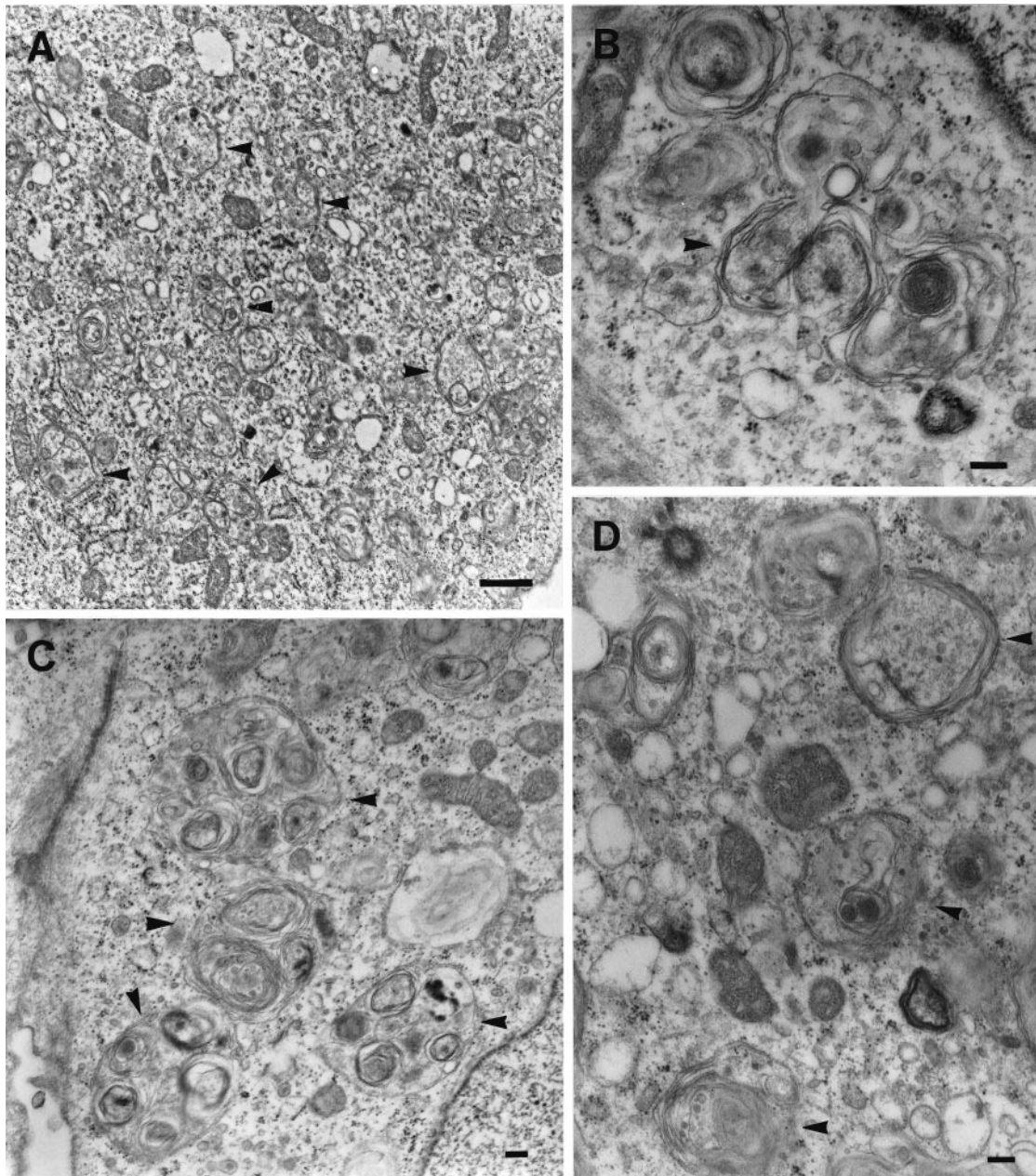


Figure 7. Expression of inclusion bodies in 3-MA-treated GlcNAc-TV transfectants. Treatment of GlcNAc-TV-transfected M1 cells with 10 mM 3-MA for 3 d resulted in the disappearance of MLBs and the appearance of morphologically distinct inclusion bodies (see arrows) exhibiting multiple external membranes and resembling AV_i (A–D). Bar, 0.5 μ m (A); 0.2 μ m (B–D).

for protein glycosylation in organelle biogenesis and in the expression of a differentiated phenotype by this lung type II-derived alveolar cell line in culture.

The immunofluorescent L-PHA labeling of the large LAMP-2-positive vacuoles localizes β 1–6-branched L-PHA substrates to MLBs. Increased L-PHA reactivity of LAMP-2 was demonstrated in M1 and M9 cells compared with untransfected Mv1Lu cells (Demetriou *et al.*, 1995). In both M9 and M1 cells, LAMP-2 migrates more slowly in SDS-PAGE

than in Mv1Lu cells, even though M1 cells exhibit increased L-PHA reactivity of LAMP-2 relative to M9 cells, corresponding to their twofold increased expression of GlcNAc-TV (Demetriou *et al.*, 1995). The basis for the increased MLB expression of M9 cells compared with M1 cells is not clear, and the specific aspect of poly-lactosamine glycosylation that induces MLB formation is not known. Direct demonstration of a role for poly-lactosamine glycosylation in MLB biogenesis proved difficult, because inhibitors of the glycosylation bio-

Table 2. 3-MA decreases multilamellar body expression

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
M1 control				
MLBs (%)	5.6 ± 1.1	8.6 ± 1.7	9.1 ± 1.6	ND
M1 + 3-MA				
MLBs (%)	0.05 ± 0.04 ^a	2.1 ± 0.1 ^b	0.08 ± 0.06 ^a	ND
Inclusion bodies (%)	3.1 ± 1.1	2.8 ± 0.6	5.5 ± 1.3	ND
M9 control				
MLBs (%)	13.7 ± 2.1	24.4 ± 4.3	12.3 ± 2.8	11.8 ± 3.5
M9 + 3-MA				
MLBs (%)	5.2 ± 1.6 ^b	10.5 ± 1.5 ^c	0.3 ± 0.3 ^a	2.3 ± 1.4 ^c
Inclusion bodies (%)	1.2 ± 0.1	1.8 ± 0.6	1.8 ± 0.5	0.5 ± 0.1

GlcNAc-TV-transfected M1 and M9 Mv1Lu clones were plated for 6 d in regular medium (control). For the 3-MA-treated cells (+ 3-MA), the culture medium was supplemented with 10 mM 3-MA for the final 3 d. The area of MLBs and of cytoplasm was quantified as for Table 1. The area covered by inclusion bodies, morphologically distinguished from MLBs (see MATERIALS AND METHODS), in the 3-MA-treated cells was also measured. Inclusion bodies were not detected in control cells. ND, not determined.

^{a-c} The significance of the difference in MLB/cytoplasm area between control and 3-MA treated cells is as follows: ^a $p < 0.005$; ^b $p < 0.01$; ^c $p < 0.05$.

synthetic pathway also inhibit the corresponding lysosomal glycosidases, thereby preventing autophagic vacuole degradation (Tulsiani and Touster, 1992). Putative β 1–6 branching and polyactosamine glycosylation of MLB glycoproteins might enhance their resistance to degradation by lysosomal proteases or modify interactions between MLB components, thereby favoring lamella formation.

Role of Lysosomal Degradation in MLB Biogenesis

The large vacuoles immunofluorescently labeled with antibodies to LAMP-2 are present predominantly in the M9 and M1 GlcNAc-TV transfectants as are morphologically identifiable MLBs by electron microscopy. The fact that no other structure comparable in size with the MLBs is present in the transfected cells identifies the large fluorescently labeled LAMP-2- and L-PHA-positive vacuoles as MLBs. Deficiency in lysosomal galactosidases and sialidases is associated with the accumulation of lamellar bodies, demonstrating that impaired lysosomal degradation of glycoproteins or glycolipids can be associated with the formation of lamellar bodies (Amano *et al.*, 1983; Alroy *et al.*, 1985; Allegranza *et al.*, 1989; Ohshima *et al.*, 1997).

A definitive role for lysosomal degradation in MLB formation was demonstrated by leupeptin treatment of GlcNAc-TV transfectants (Figures 4 and 5). Over 3–4 d, MLBs are gradually replaced by AV_d, implicating leupeptin inhibition of lysosomal proteases in the prevention of de novo formation of MLBs from AV_d. In the absence of new synthesis of MLBs, the disappearance of MLBs could occur via normal turnover mechanisms, which may include dilution caused by cell division or secretion. Lamellar membrane structures can be visualized in the extracellular space of GlcNAc-TV-transfected Mv1Lu cells (Figure 3D). However, the presence of peripheral dense regions in MLBs 15 h after addition of leupeptin is also indicative of the fusion of MLBs with endosomes and/or lysosomes whose contents are not transformed into membrane lamellae in the absence of lysosomal degradation. The appearance of heterologous transforming vacuoles after leupeptin treatment suggests that MLBs are

continually fusing with lysosomes and that transformation of newly incorporated material into membrane lamellae requires lysosomal degradation. The endocytic pathway has been shown to deliver material to nascent autophagic vacuoles, and the autophagic pathway is therefore accessible at early stages (Gordon and Seglen, 1988; Tooze *et al.*, 1990; Liou *et al.*, 1997). Fusion of lysosomes with degradative autophagic vacuoles has also been documented (Ericsson, 1969; Lawrence and Brown, 1992; Yokota *et al.*, 1995). Our data support the idea that heterologous fusion events between lysosomes and MLBs are continually occurring; whether these fusion events represent complete incorporation of the lysosome into the MLB or rather a kiss and run mechanism is not clear (Storrie and Desjardins, 1996).

The transformation of leupeptin-induced AV_d into MLBs after leupeptin washout demonstrates that lysosomal degradation is a critical element in the formation of membrane lamellae. The formation of lamellae within distinct subregions of the autophagic vacuole (Figure 5, D and E) further indicates that localized degradation is responsible for the formation of a microenvironment propitious for lamellae formation. In GlcNAc-TV-transfected Mv1Lu cells, β 1–6 branching of N-glycans of LAMPs and possibly other as yet unidentified MLB glycoproteins therefore generates a lipid-protein mix, which is conducive to the formation of membrane lamellae within the degradative lysosomal environment of the AV_d. Continuing lysosome fusion could generate large lysosomal organelles whose contents cannot be degraded by lysosomal hydrolases, resulting in the formation of a residual body of lysosomal degradation or an MLB. However, the ability to inhibit MLB formation with 3-MA, a specific inhibitor of autophagy, demonstrates that in the cell system studied here, lysosome fusion with autophagic vacuoles is necessarily involved in MLB biogenesis.

Biogenesis of MLBs via Autophagy

A specific role for autophagic sequestration in MLB biogenesis was demonstrated by the ability of 3-MA to prevent the formation of MLBs in GlcNAc-TV-transfected

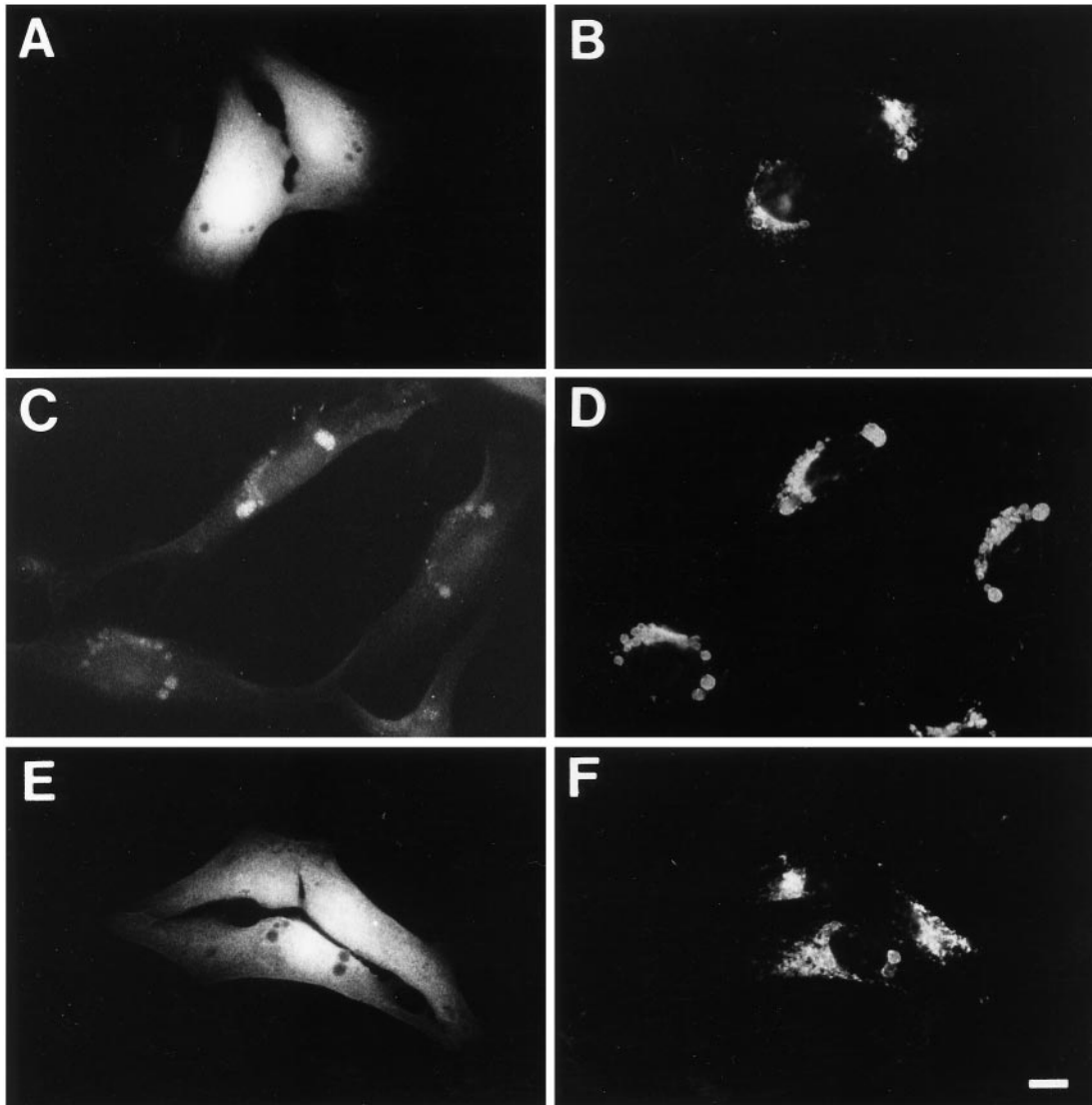


Figure 8. Incorporation of cytosolic FITC-dextran into MLBs is inhibited by 3-MA. M9 cells were scraped from plastic dishes in the presence of 2.5 mg/ml FITC-dextran, washed extensively, replated on coverslips in regular medium (A–D) or medium containing 10 mM 3-MA (E and F), and then fixed after 2 (A and B) or 48 (C–F) h and labeled for LAMP-2 using Texas Red-conjugated secondary antibodies. The distribution of FITC-dextran (A, C, and E) and LAMP-2 (B, D, and F) in the same cells is presented. After only 2 h of plating, FITC-dextran is cytosolic and excluded from large LAMP-2-positive vacuoles (A and B); however, with time FITC-dextran is incorporated via autophagy into LAMP-2-positive perinuclear vacuoles equivalent to MLBs (C and D). Autophagic incorporation of FITC-dextran into MLBs is inhibited by 3-MA (E and F). Bar, 10 μ m.

cells (Figure 6 and Table 2). 3-MA treatment is associated with increased lysosomal pH and decreased lysosomal density and with inhibition of late endosome to lysosome transport (Caro *et al.*, 1988; Punnonen *et al.*, 1994), which may explain the slight enlargement of LAMP-2-positive structures in both GlcNAc-TV-transfected and untransfected Mv1Lu cells (Figure 6, B and D). Nevertheless, 3-MA treatment of GlcNAc-TV transfectants results in the disappearance of large LAMP-2-labeled vacuoles corresponding to MLBs as well as the significant reduction in morphologically identifiable MLBs by electron micros-

copy. Inhibition of autophagy with 3-MA is therefore generally associated with the disappearance of MLBs. 3-MA treatment results in the accumulation of inclusion bodies that morphologically resemble AV_i , demonstrating that 3-MA is blocking autophagy in the GlcNAc-TV transfectants at an early stage of autophagic vacuole biogenesis. In the hepatocyte, 3-MA blocks the initial sequestration event in autophagic vacuole biogenesis and is associated with an approximate twofold reduction in autophagic sequestration (Kopitz *et al.*, 1990; Seglen and Bohley, 1992), which is consistent with the reduction (be-

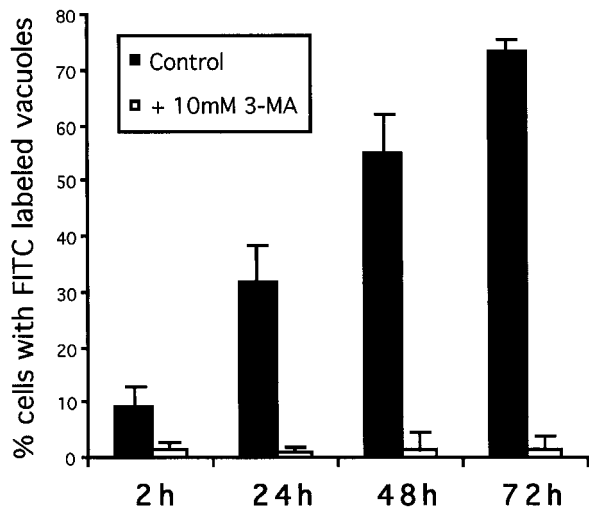


Figure 9. Autophagy of scrape-loaded FITC-dextran in GlcNAc-TV-transfected cells. FITC-dextran was scrape loaded into M9 cells as in Figure 8 and incubated in regular medium (filled bars) or medium supplemented with 10 mM 3-MA (open bars) for 2, 24, 48, or 72 h (as indicated) before fixation and labeling for LAMP-2 as in Figure 8. Fifty FITC-dextran-loaded cells per slide were assessed for the presence of FITC labeling in LAMP-2-positive vacuoles. The percent of cells that exhibit FITC labeling of lysosomal vacuoles is presented and represents the average \pm SD of six experiments.

tween 40 and 83%) in cytoplasmic area covered by both MLBs and inclusion bodies in 3-MA-treated M1 and M9 cells observed here (Table 2).

The demonstration that cytoplasmic FITC-dextran can be incorporated into the LAMP-2-positive MLBs provides a direct illustration that autophagic sequestration is involved in MLB biogenesis (Figure 8). The fact that this sequestration process is inhibited by 3-MA clearly shows that 3-MA is inhibiting autophagy in these cells and that inhibition of autophagy is directly responsible for the decreased expression of MLBs in 3-MA-treated cells. A similar approach has been recently been used to demonstrate that the parasitophorous vacuoles of *Leishmania mexicana* acquire cytosolic material via autophagy, and, in a manner similar to our results, this process is inhibited by 3-MA (Schaible *et al.*, 1999). The role of autophagic vacuole biogenesis in MLB formation implicates autophagy not only in the cellular response to stress but also in a normal cellular function, MLB formation, and surfactant secretion by the lung type II alveolar cell. The necessary role of autophagy in MLB biogenesis in Mv1Lu cells suggests that autophagy and autophagic vacuole maturation are involved in MLB biogenesis in multiple cell types. If so, our data may have significant implications for the mechanism of MLB accumulation in lysosomal storage diseases.

The formation of the multiple membrane lamella of the MLB requires a vast amount of cellular lipids, and autophagy may constitute the most efficient means of accumulating the necessary molecules within a single organelle. Select resistance of the contents of the autophagic vacuole to lysosomal degradation, possibly because of to β 1-6-branched N-glycans in this GlcNAc-TV-transfected cellular model and

other mechanisms in various cell types and pathological states, results in the localized formation of membrane lamellae, which give rise to the concentric membrane whorls of the MLB.

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REFERENCES

- Allegranza, A., Tredici, G., Marmiroli, P., di Donato, S., Franceschetti, S., and Mariani, C. (1989). Sialidosis type I: pathological study in an adult. *Clin. Neuropathol.* 8, 266–271.
- Alroy, J., Orgad, U., Ucci, A.A., Schelling, S.H., Scunk, K.L., Warren, C.D., Raghavan, S.S., and Kolodny, E.H. (1985). Neurovisceral and skeletal gangliosidosis in dogs with beta-galactosidase deficiency. *Science* 229, 470–472.
- Amano, N., Yokoi, S., Akagi, M., Sakai, M., Yagashita, S., and Nakata, K. (1983). Neuropathological findings of an autopsy case of adult β -galactosidase and neuraminidase deficiency. *Acta Neuropathol.* 61, 283–290.
- Amos, B., and Lotan, R. (1990). Modulation of lysosomal-associated membrane glycoproteins during retinoic acid-induced embryonal carcinoma cell differentiation. *J. Biol. Chem.* 265, 19192–19198.
- Balis, J.U., and Conen, P.E. (1964). The role of alveolar inclusion bodies in the developing lung. *Lab. Invest.* 13, 1215–1229.
- Brockhausen, I., Romero, P.A., and Herscovics, A. (1991). Glycosyltransferase changes upon differentiation of CaCo-2 human colonic adenocarcinoma cells. *Cancer Res.* 51, 3136–3142.
- Caro, L.H., Plomp, P.J., Wolvetang, E.J., Kerkhof, C., and Meijer, A.J. (1988). 3-Methyladenine, an inhibitor of autophagy, has multiple effects on metabolism. *Eur. J. Biochem.* 175, 325–329.
- Chevalier, G., and Collet, A.J. (1972). In vivo incorporation of choline- 3 H, leucine- 3 H and galactose- 3 H in alveolar type II pneumocytes in relation to surfactant synthesis. A quantitative radioautographic study in mouse by electron microscopy. *Anat. Rec.* 174, 289–310.
- de Vries, A.C.J., Schram, A.W., Tager, J.M., Batenburg, J.J., and van Golde, L.M.G. (1985). A specific α -glucosidase in lamellar bodies of the human lung. *Biochim. Biophys. Acta* 837, 230–238.
- Demetriou, M., Nabi, I.R., Capaleno, M., Dedhar, S., and Dennis, J.W. (1995). Serum-independent growth, apoptosis and reduced substrate adhesion in epithelial cells expressing GlcNAc-transferase V. *J. Cell Biol.* 130, 383–392.
- DiAugustine, R.P. (1974). Lung concentric laminar organelle. Hydrolase activity and compositional analysis. *J. Biol. Chem.* 249, 584–593.
- Diglio, C.A., and Kikkawa, Y. (1977). The type II epithelial cells of the lung. IV. Adaptation and behavior of isolated type II cells in culture. *Lab. Invest.* 37, 622–631.
- Dobbs, L.G., Williams, M.C., and Brandt, A.E. (1985). Changes in biochemical characteristics and pattern of lectin binding of alveolar type II cells with time in culture. *Biochim. Biophys. Acta* 846, 155–166.
- Dunn, W.A. (1994). Autophagy and related mechanisms of lysosome-mediated protein degradation. *Trends Cell Biol.* 4, 139–142.
- Dunn, W.A.J. (1990). Studies on the mechanism of autophagy: formation of the autophagic vacuole. *J. Cell Biol.* 110, 1923–1935.

- Ericsson, J.L.E. (1969). Studies on induced cellular autophagy. I. Electron microscopy of cells with in vivo labeled lysosomes. *Exp. Cell Res.* 55, 95–106.
- Flaks, B., and Flaks, A. (1972). Electron-microscope observations on the formation of the cytoplasmic lamellar inclusion bodies in murine pulmonary tumors induced in vitro. *J. Pathol.* 108, 211–217.
- Fukuda, M. (1985). Cell surface glycoconjugates as onco-differentiation markers in hematopoietic cells. *Biochim. Biophys. Acta* 780, 119–150.
- Furuno, K., Ishikawa, T., Akasaki, K., Lee, S., Nishimura, Y., Tsuji, H., Himeno, M., and Kato, K. (1990). Immunocytochemical study of the surrounding envelope of autophagic vacuoles in cultured rat hepatocytes. *Exp. Cell Res.* 189, 261–268.
- Furuno, K., Ishikawa, T., and Kato, K. (1982). Isolation and characterization of autolysosomes which appeared in rat liver after leupeptin treatment. *J. Biochem.* 91, 1943–1950.
- Goldfischer, S., Kikkawa, Y., and Hoffman, L. (1968). The demonstration of acid hydrolase activities in the inclusion bodies of type II alveolar cells and other lysosomes in the rabbit lung. *J. Histochem. Cytochem.* 16, 102–109.
- Gordon, P.B., and Seglen, P.O. (1988). Prelysosomal convergence of autophagic and endocytic pathways. *Biochem. Biophys. Res. Commun.* 151, 40–47.
- Haagman, H.P., and van Golde, L.M.G. (1991). Synthesis and assembly of lung surfactant. *Annu. Rev. Physiol.* 53, 441–464.
- Hakamori, S.I. (1989). Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. *Adv. Cancer Res.* 52, 257–331.
- Hatasa, K., and Nakamura, T. (1965). Electron microscopic observations of lung alveolar epithelial cells of normal young mice, with special reference to formation and secretion of osmophilic lamellar bodies. *Z. Zellforsch.* 68, 266–277.
- Heath, M.F., Gandy, G., and Jacobson, W. (1976). Lysosomes in the lung. In: *Lysosomes in Biology and Pathology*, ed. J.T. Dingle and R.T. Dean, Amsterdam: Elsevier/North Holland, 33–58.
- Heffernan, M., Yousefi, S., and Dennis, J.W. (1989). Molecular characterization of P2B/LAMP-1, a major protein target of a metastasis-associated oligosaccharide structure. *Cancer Res.* 49, 6077–6084.
- Hoffman, L. (1972). Isolation of inclusion bodies from rabbit lung parenchyma. *J. Cell Physiol.* 79, 65–72.
- Holmes, E.H., Hakamori, S., and Ostrander, G.K. (1987). Synthesis of type 1 and 2 lacto series glycolipid antigens in human colonic adenocarcinoma and derived cell lines is due to activation of a normally unexpressed $\beta_{1-3}N$ -acetylglucosaminyltransferase. *J. Biol. Chem.* 262, 15649–15658.
- Hook, G.E.R., and Gilmore, L.B. (1982). Hydrolases of pulmonary lysosomes and lamellar bodies. *J. Biol. Chem.* 257, 9211–9220.
- Kopitz, J., Kisen, G.Ø., Gordon, P.B., Bohley, P., and Seglen, P.O. (1990). Nonselective autophagy of cytosolic enzymes in isolated rat hepatocytes. *J. Cell Biol.* 111, 941–953.
- Kovacs, A.L., Reith, A., and Seglen, P.O. (1982). Accumulation of autophagosomes after inhibition of hepatocytic protein degradation by vinblastine, leupeptin, or a lysomotropic amine. *Exp. Cell Res.* 137, 191–201.
- Lawrence, B.P., and Brown, W.J. (1992). Autophagic vacuoles rapidly fuse with preexisting lysosomes in cultured hepatocytes. *J. Cell Sci.* 102, 515–526.
- Lee, N., Wang, W.-C., and Fukuda, M. (1990). Granulocytic differentiation of HL-60 cells is associated with increase of poly-N-acetyllactosamine in Asn-linked oligosaccharides attached to human lysosomal membrane glycoproteins. *J. Biol. Chem.* 265, 20476–20487.
- Liou, W., Geuze, H.J., Geelen, J.H., and Slot, J.W. (1997). The autophagic and endocytic pathways converge at the nascent autophagic vacuole. *J. Cell Biol.* 136, 61–70.
- McNeil, P.L., Murphy, R.F., Lanni, F., and Taylor, D.L. (1984). A method for incorporating macromolecules in adherent cells. *J. Cell Biol.* 98, 1556–1564.
- Nabi, I.R., and Dennis, J.W. (1998). The extent of polyactosamine glycosylation of MDCK LAMP-2 is determined by its Golgi residence time. *Glycobiology* 8, 947–953.
- Nabi, I.R., Le Bivic, A., Fambrough, D., and Rodriguez-Boulan, E. (1991). An endogenous MDCK lysosomal membrane glycoprotein is targeted basolaterally before delivery to lysosomes. *J. Cell Biol.* 115, 1573–1584.
- Nabi, I.R., and Rodriguez-Boulan, E. (1993). Increased LAMP-2 polyactosamine glycosylation is associated with its slower Golgi transit during establishment of a polarized MDCK epithelial monolayer. *Mol. Biol. Cell* 4, 627–635.
- Ohshima, T., *et al.* (1997). α -Galactosidase A deficient mice: a model for Fabry disease. *Proc. Natl. Acad. Sci. USA* 94, 2540–2544.
- Punnonen, E.-L., Marjomäki, V.S., and Reunanen, H. (1994). 3-Methyladenine inhibits transport from late endosomes to lysosomes in cultured rat and mouse fibroblasts. *Eur. J. Cell Biol.* 65, 14–25.
- Ryan, U.S., Ryan, J.W., and Smith, D.S. (1975). Alveolar type II cells: studies on the mode of release of lamellar bodies. *Tissue Cell* 7, 587–599.
- Schaible, U.E., Schlesinger, P.H., Steinberg, T.H., Mangel, W.F., Kobayashi, T., and Russel, D.G. (1999). Parasitophorous vacuoles of *Leishmania mexicana* acquire macromolecules from the host cytosol via two independent routes. *J. Cell Sci.* 112, 681–693.
- Schmitz, G., and Müller, G. (1991). Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. *J. Lipid Res.* 32, 1539–1570.
- Seglen, P.O., and Bohley, P. (1992). Autophagy and other vacuolar protein degradation mechanisms. *Experientia* 48, 158–172.
- Seglen, P.O., and Gordon, P.B. (1982). 3-Methyladenine: a specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc. Natl. Acad. Sci. USA* 79, 1889–1892.
- Sorokin, S.P. (1967). A morphologic and cytochemical study of the great alveolar cell. *J. Histochem. Cytochem.* 14, 884–897.
- Spillmann, D., and Finne, J. (1987). Poly-N-acetyllactosamine glycans of cellular glycoproteins: predominance of linear chains in mouse neuroblastoma and rat pheochromocytoma cell lines. *J. Neurochem.* 49, 874–883.
- Storrie, B., and Desjardins, M. (1996). The biogenesis of lysosomes: is it a kiss and run, continuous fusion and fission process? *Bioessays* 18, 895–903.
- Stratton, C.J. (1978). The ultrastructure of multilamellar bodies and surfactant in the human lung. *Cell Tissue Res.* 193, 219–229.
- Tooze, J., Hollinshead, M., Ludwig, T., Howell, K., Hoflack, B., and Kern, H. (1990). In exocrine pancreas, the basolateral endocytic pathway converges with the autophagic pathway immediately after the early endosome. *J. Cell Biol.* 111, 329–345.
- Tulsiani, D.R., and Touster, O. (1992). Evidence that swainsonine pretreatment of rats leads to the formation of autophagic vacuoles and endosomes with decreased capacity to mature to, or fuse with, active lysosomes. *Arch. Biochem. Biophys.* 296, 556–561.
- Tuo, X.H., Itai, S., Nishikata, J., Mori, T., Tanaka, O., and Kannagi, R. (1992). Stage-specific expression of cancer-associated type 1 and type 2 chain polyactosamine antigens in the developing pancreas of human embryos. *Cancer Res.* 52, 5744–5751.

- Ueno, T., Muno, D., and Kominami, E. (1991). Membrane markers of endoplasmic reticulum preserved in autophagic vacuolar membranes isolated from leupeptin-administered rat liver. *J. Cell Biol.* 266, 18995–18999.
- Voorhut, W.F., Veenendahl, T., Haagsman, H.P., Weaver, T.E., Whitsett, J.A., Van Golde, L.M.G., and Geuze, H.J. (1992). Intracellular processing of pulmonary surfactant protein B in an endosomal/lysosomal compartment. *Am. J. Physiol.* 263, L479–L486.
- Voorhut, W.F., Weaver, T.E., Haagsman, H.P., Geuze, H.J., and van Golde, L.M.G. (1993). Biosynthetic routing of pulmonary surfactant proteins in alveolar type II cells. *Microsc. Res. Tech.* 26, 366–373.
- Wang, W.-C., Lee, N., Aoki, D., Fukuda, M., and Fukuda, M. (1991). The poly-*N*-acetylactosamines attached to lysosomal membrane glycoproteins are increased by prolonged association with the Golgi complex. *J. Biol. Chem.* 266, 23185–23190.
- Williams, M.C. (1977). Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs. *J. Cell Biol.* 72, 260–277.
- Yamashita, K.Y., Tachibana, Y., Ohkura, T., and Kobata, A. (1985). Enzymatic basis for the structural changes of asparagine-linked sugar chains of membrane glycoproteins of baby hamster kidney cells induced by polyoma virus. *J. Biol. Chem.* 260, 3963.
- Yokota, S., Himeno, M., and Kato, K. (1995). Formation of autophagosomes during degradation of excess peroxisomes induced by di-(2-ethylhexyl)-phthalate treatment. III. Fusion of early autophagosomes with lysosomal compartments. *Eur. J. Cell Biol.* 66, 15–24.
- Youakim, A., Romero, P.A., Yee, K., Carlsson, S.R., Fukuda, M., and Herscovics, A. (1989). Decrease in polyactosamines associated with lysosomal membrane glycoproteins during differentiation of CaCo-2 human colonic adenocarcinoma cells. *Cancer Res.* 49, 6889–6895.
- Yousefi, S., Higgins, E., Daoling, Z., Pollex-Kruger, A., Hindsgaul, O., and Dennis, J.W. (1991). Increased UDP-GlcNAc:Gal β ₁₋₃GalNAc-R (GlcNAc to GalNAc) β -1,6-*N*-acetylglucosaminyltransferase activity in metastatic murine tumor cell lines. Control of polyactosamine synthesis. *J. Biol. Chem.* 266, 1772–1782.