A role for the lysosomal membrane protein LGP85 in the biogenesis and maintenance of endosomal and lysosomal morphology

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Summary

LGP85 (LIMP II) is a type III transmembrane glycoprotein that is located primarily in the limiting membranes of lysosomes and late endosomes. Despite being the abundant molecule of these compartments, whether LGP85 merely resides as one of the constituents of these membranes or plays a role in the regulation of endosome and lysosome biogenesis remains unclear. To elucidate these questions, we examined the effects of overexpression of LGP85 on the morphology and membrane traffic of the endosomal/lysosomal system. Here we demonstrate that overexpression of LGP85 causes an enlargement of early endosomes and late endosomes/lysosomes. Such a morphological alteration was not observed by overexpression of other lysosomal membrane proteins, LGP107 (LAMP-1) or LGP96 (LAMP-2), reflecting a LGP85-specific function. We further demonstrate that overexpression of LGP85 impairs the endocytic membrane traffic out of these enlarged compartments, which may be correlated with or account for the accumulation of cholesterol observed in these compartments. Interestingly, co-transfection of LGP85 and the dominant-negative form of Rab5b (Rab5bS34N) abolished the formation of large vacuoles, suggesting that the GTP-bound active form of Rab5b is involved in the enlargement of endosomal/lysosomal compartments induced by overexpression of LGP85. Thus, these findings provide important new insights into the role of LGP85 in the biogenesis and the maintenance of endosomes/lysosomes. We conclude that LGP85 may participate in reorganizing the endosomal/lysosomal compartments.

Key words: LGP85, Lysosome, Membrane traffic, Rab5

Introduction

Lysosomes are acidic membrane-bound organelles involved in degradation of extracellular materials internalized by endocytosis and intracellular materials derived from within cells by autophagy (de Duve, 1983; Kornfeld and Mellman, 1989; Hunziker and Geuze, 1996). The formation of lysosomes requires protein transport from the biosynthetic and the endocytic pathway. Proteins destined to be targeted to lysosomes are delivered to an acidic endosomal compartment either from the trans-Golgi network (TGN) or from the plasma membrane via endocytosis. Following delivery to endosomes, certain receptor molecules are segregated from proteins that reside in lysosomes and recycle back to the TGN or to the plasma membrane (van Deurs et al., 1993; Gruenberg and Maxfield, 1995; Futter et al., 1996). During this process, a subset of membrane proteins targeted to lysosomes for degradation is sorted into vesicles that invaginate from the limiting membrane of endosomes. This process forms a multivesicular body (MVB), the late endosome, which subsequently fuses directly with lysosomes (Futter et al., 1996; Bright et al., 1997). By contrast, proteins destined for the limiting membrane of lysosomes remain in the limiting membrane of the MVB.

It has been considered that lysosomal membrane proteins have multiple functions, such as sequestration of numerous acid hydrolases, maintenance of an acidic intralysosomal environment, transport of degradation products (amino acids and carbohydrates) from the lysosomal lumen to the cytoplasm, and specific interaction and fusion between lysosomes and other organelles (Fukuda, 1991; Peters and von Figura, 1994; Hunziker and Geuze, 1996). The limiting membrane of lysosomes contains a characteristic set of highly glycosylated transmembrane proteins. Based on their amino acid sequences deduced from cDNA cloning of different species, five major membrane proteins, LAMP-1, LAMP-2, LAMP-3 (also known as LIMP I or CD63), LGP85 (LIMP II) and lysosomal acid phosphatase, have been identified. Among them, LAMP-1 and LAMP-2 are the best characterized lysosomal membrane proteins; they contain a large luminal domain bearing a number of N-linked oligosaccharide chains, a single transmembrane anchor, and a short cytoplasmic domain bearing a tyrosine-based motif, which is necessary and sufficient for their targeting to lysosomes (Williams and

Fukuda, 1990; Harter and Mellman, 1992; Guarnieri et al., 1993; Höning and Hunziker, 1995; Gough and Fambrough, 1997; Gough et al., 1999).

LGP85 (LIMP II), one of the major lysosomal membrane proteins (Barrinocanal et al., 1986; Okazaki et al., 1992), is characterised by a different structure. Unlike LAMP-1 and LAMP-2, which are type I membrane proteins, LGP85 is a type III membrane protein that is inserted into the intracellular membrane by an N-terminal uncleavable signal sequence and a short C-terminal domain (Vega et al., 1991; Fujita et al., 1991; Fujita et al., 1992). In addition, the signal responsible for targeting of LGP85 to lysosomes is also different. Instead of a tyrosine-based motif, a di-leucine-based motif present in the C-terminal cytoplasmic domain mediates effective lysosomal targeting (Ogata and Fukuda, 1994; Sandoval et al., 1994).

So far, two different pathways for transport of newly synthesized lysosomal membrane proteins have been proposed (reviewed by Hunziker and Geuze, 1996). One of them is the direct intracellular route that delivers these proteins from the TGN to lysosomes via endosomes. The other is the indirect route in which newly synthesized lysosomal membrane proteins are transported from the TGN to the plasma membrane and subsequently delivered to lysosomes via the endocytic pathway. In both, direct and indirect intracellular routes, trafficking and sorting of lysosomal membrane proteins are mediated by distinct heterotetrameric adaptor complexes. Four adaptor complexes, AP-1, AP-2, AP-3, and AP-4, and GGAs have been described and are thought to function in the transport of cargo proteins into the endocytic and lysosomal pathways (reviewed by Kirchhausen, 1999; Robinson and Bonifacino, 2001). Recent studies have pointed to a participation of AP-3 in sorting of several lysosomal membrane proteins including LAMPs and LGP85 (LIMP II) to lysosomes from the TGN or endosomes using both tyrosine- and di-leucine-based sorting motifs (Höning et al., 1998; Le Borgne et al., 1998; Dell'Angelica et al., 1999).

Several lines of evidence in vivo and in vitro have revealed that late endosomes and lysosomes undergo multiple cycles of fusion and fission (Storrie and Desjardins, 1996; Futter et al., 1996; Bright et al., 1997; Mullock et al., 1998; Luzio et al., 2000). This implies that late endosomes and lysosomes are in dynamic equilibrium with each other (Mellman, 1996). This could explain why all lysosomal membrane proteins reside both in late endosomes and lysosomes. In vitro fusion experiments have identified several different molecules, such as NSF, SNAPs and Rab GDI-sensitive GTPase, to be involved in fusion between late endosomes and lysosomes (Mullock et al., 1998). However, the precise molecular mechanisms responsible for late endosome-lysosome fusion have not yet been established. Despite the extensive knowledge regarding intracellular traffic of lysosomal membrane proteins, there is still no direct evidence whether lysosomal membrane proteins are involved in membrane traffic between late endosomes and lysosomes. If overexpression of lysosomal membrane proteins causes morphological changes in late endosomes and/or lysosomes by perturbing membrane equilibrium between these compartments, it could be possible that lysosomal membrane proteins themselves are involved in the membrane traffic between late endosomes and lysosomes – directly or indirectly – as components of a machinery regulating docking/fusion or fission.

In the present work, we have overexpressed several lysosomal membrane proteins in COS cells and analyzed their effect on the morphology and membrane traffic of the endosomal/lysosomal system. We show that overexpression of LGP85 specifically results in large swollen vacuoles, which have characteristics of endosomes and lysosomes. We also show perturbation of membrane traffic out of, and accumulation of free-cholesterol in, these enlarged endosomal and lysosomal compartments. Together with an inhibitory effect of the dominant-negative form of Rab5b on the formation of large vacuoles induced by overexpression of LGP85, we conclude that LGP85 may be involved in the biogenesis of endosomes/lysosomes and in the reorganization of the endosomal/lysosomal compartments, presumably through the interaction with a machinery regulating vesicular fusion or fission.

Materials and Methods

Materials

Culture media and fetal calf serum were purchased from Gibco BRL (Grand Island, NY). Filipin and lipoprotein-deficient bovine serum (LPDS) were obtained from Sigma (St Louis, MO). Alexa-594-conjugated human transferrin (Tfn), epidermal growth factor (EGF), biotinylated, complexed to Texas Red streptavidin, Texas Red-conjugated dextran (M_r 70,000 lysine fixable), and Alexa-488-, Alexa-594-, and Cascade Blue-labeled secondary antibodies were purchased from Molecular Probes (Eugen, OR). FuGENE 6 was from Roche Molecular Biochemicals (Indianapolis, IN). pcDNA3.1 was obtained from Invitrogen (Groningen, The Netherlands).

Antibodies

Rabbit polyclonal antibodies to rat LGP85 were raised against the purified LGP85 from isolated rat liver lysosomal membranes (Okazaki et al., 1992). Mouse monoclonal antibodies to rat LGP85 and LGP96 were kindly provided from Kenji Akasaki (Fukuyama University, Japan). Rabbit polyclonal antibodies to rat LGP107 have been previously described (Furuno et al., 1989a). Mouse monoclonal antibodies to human LAMP-2 were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA). Mouse monoclonal antibodies to early endosome autoantigen 1 (EEA1) and GM130 were purchased from Transduction Laboratories (Lexington, KY). Mouse monoclonal antibodies to human Tfn receptor (TfnR) were obtained from Zymed Laboratories (San Francisco, CA). Mouse monoclonal antibodies to FLAG M2 were purchased from Sigma. Mouse monoclonal antibodies to lysobisphosphatidic acid (LBPA) were a kind gift of Toshihide Kobayashi (RIKEN, Tokyo). Rabbit polyclonal antibodies to rat cation-independent mannose 6-phosphate/insulin-growth factor II receptor (MPR300) were raised against a fusion protein encoding glutathion S-transferase coupled to a portion of the cytoplasmic tail (amino acids 2313 to 2409) of rat MPR300 (MacDonald et al., 1988).

Cell culture and transfection

COS-1, HeLa, MDCK, NRK and NIH3T3 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, and 100 units of penicillin/streptomycin/ml in humidified 95% air and 5% CO_2 at 37°C. The cells were plated onto 13 mm coverslips the day before transfection. The full-length rat LGP85 (Fujita et al., 1991), rat LGP107 (Himeno et al., 1989), or rat LGP96 (Noguchi et al., 1989) cDNA was then inserted into the expression vector pcDNA3.1 (Invitrogen). The human wild-type Rab5b, Rab5bQ79L, and

Rab5bS34N cDNAs inserted into the pCMV5-Flag vector (Kurosu and Katada, 2001) were kindly provided from Kota Saito and Toshiaki Katada (Tokyo University, Japan). The transfections were carried out with FuGENE6, according to the manufacturer's instructions. After 36 hours the cells were used for immunocytochemical experiments.

Immunofluorescence microscopy

Cells cultured on coverslips and transfected with LGP85 were rinsed with phosphate-buffered saline (PBS), fixed immediately in 4% paraformaldehyde (PFA) in PBS, pH 7.4, for 15 minutes at room temperature, and permeabilized with 0.05% saponin in PBS for 15 minutes or methanol for 5 minutes at 0°C (for EEA1 immunostaining). To label lysosomes, cells cultured on coverslips were incubated with Texas Red-labeled dextran (1 mg/ml) in serum-free DMEM with 1 mg/ml BSA for 4 hours at 37°C and then cultured in normal medium for 20 hours. After that, cells were transfected with LGP85, fixed in 4% PFA in PBS at 12, 24 or 36 hours post-transfection, and permeabilized with 50 μg/ml digitonin in PBS. Cells were quenched with 50 mM NH_4Cl in PBS for 15 minutes and blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes. The cells were then incubated for 1 hour in the primary antibody diluted in blocking solution using the following dilutions of the primary antibodies: anti-LGP85 antibody (1:3000), anti-LAMP-2 antibody (1:100), anti-EEA1 antibody (1:100), anti-LBPA antibody (1:100), anti-MPR300 antibody (1:300), anti-GM130 antibody (1:100), and anti-FLAG antibody (1:300). The cells were washed with blocking solution and incubated for 30 minutes with the secondary antibodies diluted in blocking solution. Coverslips were then washed three times with blocking solution, rinsed with water, and mounted onto glass slides. Visualization of acidic compartments by 3-(2,4-dinitroanilino)-3′-amino-N-methyldipropylamine (DAMP) was carried out using the acidic granule kit (Oxford Biomedical Research, MI) according to the manufacturer's instructions. Fluorescence was viewed using a fluorescence light microscope (Leica DMRB; Wetzlar, Germany) and a 63× oil immersion lens. Photographic images were acquired using a cooled CCD camera (MicroMAX; Princeton Instruments, Trenton, NJ), processed using IPlab software (Scanalytics, Fairfax, VA), and merged using Adobe Photoshop software (Adobe Systems, Mountain View, CA). For some experiments, the cells were analyzed by confocal laser scanning microscopy using a Radiance 2100 MP confocal microscope (Bio-Rad Laboratories, Richmond, CA) with an argon/krypton laser, the Red Diode laser, and the Blue Diode laser.

Tfn, EGF and dextran internalization

For Tfn internalization, cells cultured on coverslips and transfected with LGP85 were incubated in serum-free DMEM with 1 mg/ml BSA for 30 minutes and then with Alexa-594-labeled Tfn (50 μg/ml) for 30 minutes at 37°C. For EGF internalization, cells cultured on coverslips and transfected with LGP85 were incubated in serum-free DMEM with 1 mg/ml BSA for 12 hours and then with EGF, biotinylated, complexed to Texas Red streptavidin (3.3 μg/ml) for 1 hour at 4°C. Cells were then shifted to 37°C for 3 hours to allow internalization. For dextran internalization, cells cultured on coverslips and transfected with LGP85 were washed with serum-free DMEM with 1 mg/ml BSA and then incubated with Texas Red-labeled dextran (1 mg/ml) for 30 minutes at 37°C. After a chase of 3 hours, cells were washed with PBS, fixed in 4% PFA in PBS, and permeabilized with 50 μg/ml digitonin in PBS, followed by immunostaining for LGP85, EEA1, or LAMP-2.

Filipin staining

Cells cultured on coverslips and transfected with LGP85 were rinsed with PBS, fixed in 4% PFA in PBS, pH 7.4, for 30 minutes at room temperature. Cells were stained (and permeabilized) with 0.05% filipin in PBS for 1 hour, followed by immunostaining for LGP85.

Immunoelectron microscopy

Cells were cultured on 10 cm plastic dishes and transfected with LGP85. Monolayers were fixed in 4% paraformaldehyde in 0.2 M Hepes, pH 7.4 for 2 hours at room temperature, and then stored in 2% paraformaldehyde in the same buffer. The cells were embedded in 10% gelatin, infiltrated with 17% polyvinylpyrrolidone-1.8 M sucrose, and frozen in liquid nitrogen. Thin frozen sections were cut at –100°C and picked up with sucrose-methyl cellulose. The sections were immunolabelled with rabbit anti-LGP85 mixed with either mouse anti-LAMP-2 or LBPA, followed by a mixture of goat anti-rabbit-10 nm gold and goat anti-mouse 5 nm gold (British BiCell, Cardiff, UK).

Results

Expression of rat LGP85 in COS cells causes the formation of large swollen vacuoles

The cDNA encoding rat LGP85 was inserted into an expression vector, pcDNA3.1, and was transiently transfected into COS cells. Thirty-six hours after transfection, cells were fixed and intracellular localization of LGP85 visualized by indirect immunofluorescence microscopy. The antibodies raised against rat LGP85 did not recognize endogenous LGP85 in COS cells. Interestingly, expression of LGP85 resulted in the appearance of numerous large swollen vacuoles throughout the cytoplasm, all of which were stained with LGP85 antibody (Fig. 1A). Moreover, these large swollen vacuoles were also easily seen as phase lucent vacuoles by phase contrast microscopy (Fig. 1B) which, therefore, makes it easy to distinguish transfected cells from untransfected cells. Under the conditions used, LGP85 was expressed in more than 80% of cells, and approximately 80% of the positive cells displayed large swollen vacuoles, while the remainder exhibited small punctate structures. In cells with large swollen vacuolar staining, weak staining of the nuclear envelope and the ER could be sometimes observed. It seems likely that large swollen vacuoles could be formed only in cells overexpressing LGP85. The size of the vacuoles varied among individual cells, ranging from 2-10 μm depending on the time after transfection. The formation of large swollen vacuoles induced by overexpression of LGP85 was also observed in HeLa (Fig. 1C,D), MDCK cells (Fig. 1E,F) as well as U251, NRK and NIH3T3 cells (data not shown). However, the size of vacuoles and efficiency of transfection varied in cell types (Fig. 1). Since the transfection efficiency was much higher than in other cell lines, we used COS cells in all following experiments.

Appearance of large vacuoles is specific to expression of LGP85

We examined whether the appearance of swollen vacuoles is specific to expression of LGP85. To this end, either LGP107 (rat LAMP-1) or LGP96 (rat LAMP-2) cDNA, both of which are well known as late endosome/lysosome markers (Furuno et al., 1989a; Furuno et al., 1989b), was expressed in COS cells. We have previously isolated lysosomal membranes from rat liver (Ohsumi et al., 1983). We analyzed them on SDS-PAGE and Coomassie blue staining and found that LGP107 and

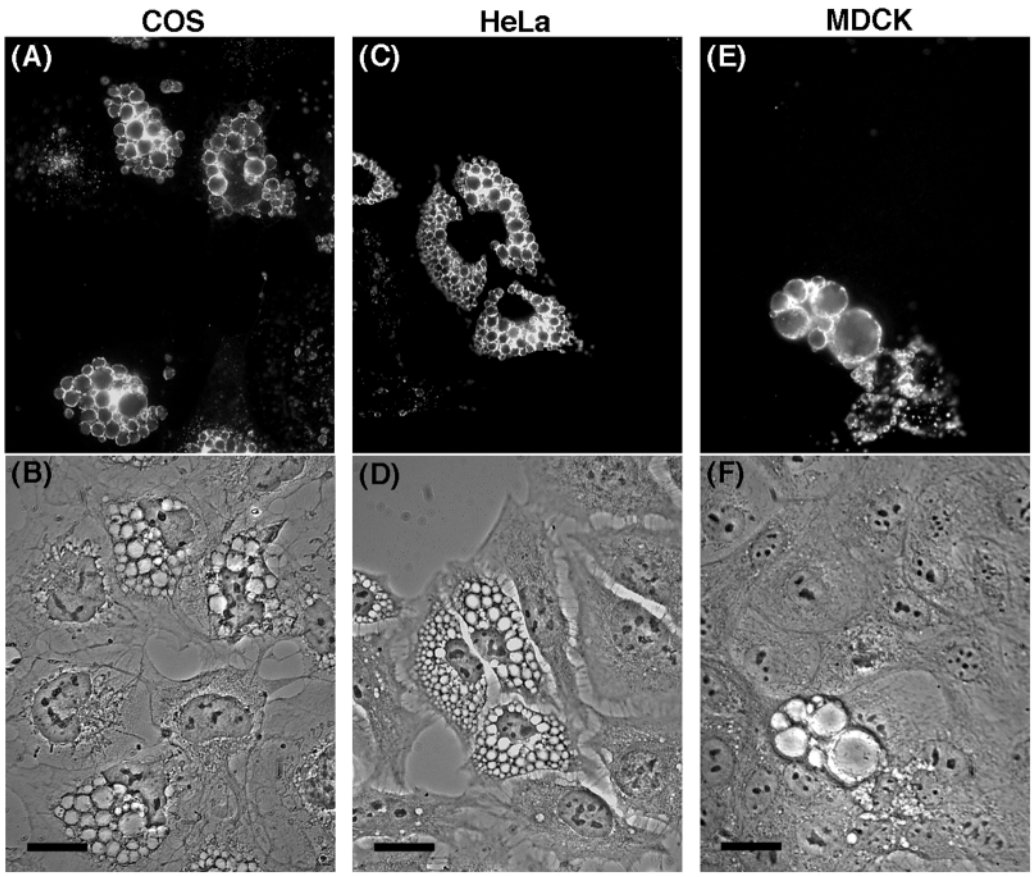

Fig. 1. Overexpression of LGP85 in COS cells causes large swollen vacuoles. Cultured COS (A,B), HeLa (C,D), or MDCK (E,F) cells were transiently transfected with LGP85 and after 36 hours were fixed and processed for immunofluorescence micrography using anti-LGP85 polyclonal antibody as described in Materials and Methods. Upper panels (A,C,E) show the LGP85 staining and the lower panels (B,D,F) show the corresponding phase-contrast micrographs. Bars, 20 μm.

LGP96 are the most abundant proteins of the lysosomal membrane. Based on our results, we consider the relative abundance of lysosomal membrane proteins to be the following: LGP107=LGP96>LGP85>acid phosphatase> LAMP-3. In contrast to LGP85, large swollen vacuoles were never observed in cells expressing LGP107 or LGP96 (Fig. 2), indicating that the formation of large swollen vacuoles is specific to expression of LGP85. Essentially, identical results were obtained using other cell types described above (data not shown).

Characterization of large vacuolar compartments induced by overexpression of LGP85

To clarify the characteristics of LGP85-induced large swollen vacuoles, we carried out double labeling with several well-characterized organelle markers. LAMP-2, which localizes in both late endosomes and lysosomes, was predominantly detected on the limiting membrane of LGP85-positive swollen large vacuoles (Fig. 3A-C). However, LAMP-2 staining did not completely overlap with LGP85; some LGP85-positive large vacuoles were always negative for LAMP-2. Similar results were obtained with LBPA, a late endosome-specific marker (Kobayashi et al., 1998). Although LBPA is known to be enriched in internal vesicles of the late endosome, LBPA staining was predominantly found as punctate structures in close proximity to or overlapping with LGP85 within or in the vicinity of the limiting membrane of large vacuoles (Fig. 3D-F). EEA1, a marker for the early endosome (Mu et al., 1995), was localized as small punctate structures throughout the cytoplasm in untransfected cells. By contrast, expression of LGP85 resulted in an alteration of EEA1 distribution; most of EEA1 colocalized with LGP85 on the limiting membrane of large vacuoles (Fig. 3G-I). In this case, however, only a few LGP85-positive large vacuoles that were located predominantly near the perinuclear region were stained with EEA1 antibodies. TfnR, an early and recycling endosomes marker, was also observed in a few LGP85-positive large vacuoles (Fig. 3J-L) as seen with EEA1 staining. In contrast to these endosomal and lysosomal markers, MPR300, which localizes in the TGN and late endosomes and cycles between these compartments (Griffith et al., 1988; Stoorvogel et al., 1991; Kornfeld, 1992), was distributed predominantly in the perinuclear region and was not seen in the LGP85-induced large vacuoles (Fig. 3M-O). Although MPR300 has also been used as a TGN/late endosome marker, MPR300 never overlapped with LBPA nor LAMP-2, even in untransfected cells, suggesting that MPR300 localizes mainly in the TGN in COS cells. This notion was further supported by the observation that treatment of cells with BFA, a drug that is known to cause extensive tubulation of the TGN and early endosomes (Lippincott-Schwartz et al., 1991; Wood and Brown, 1992), altered perinuclear distribution of MPR300 to tubular staining pattern (data not shown). Similarly, syntaxin 6, a TGN marker (Bock et al., 1997) was not localized in large swollen vacuoles induced by overexpression of LGP85 (data not shown). GM130, which localizes in cis-Golgi (Nakamura et al., 1995), showed a relatively compact staining pattern

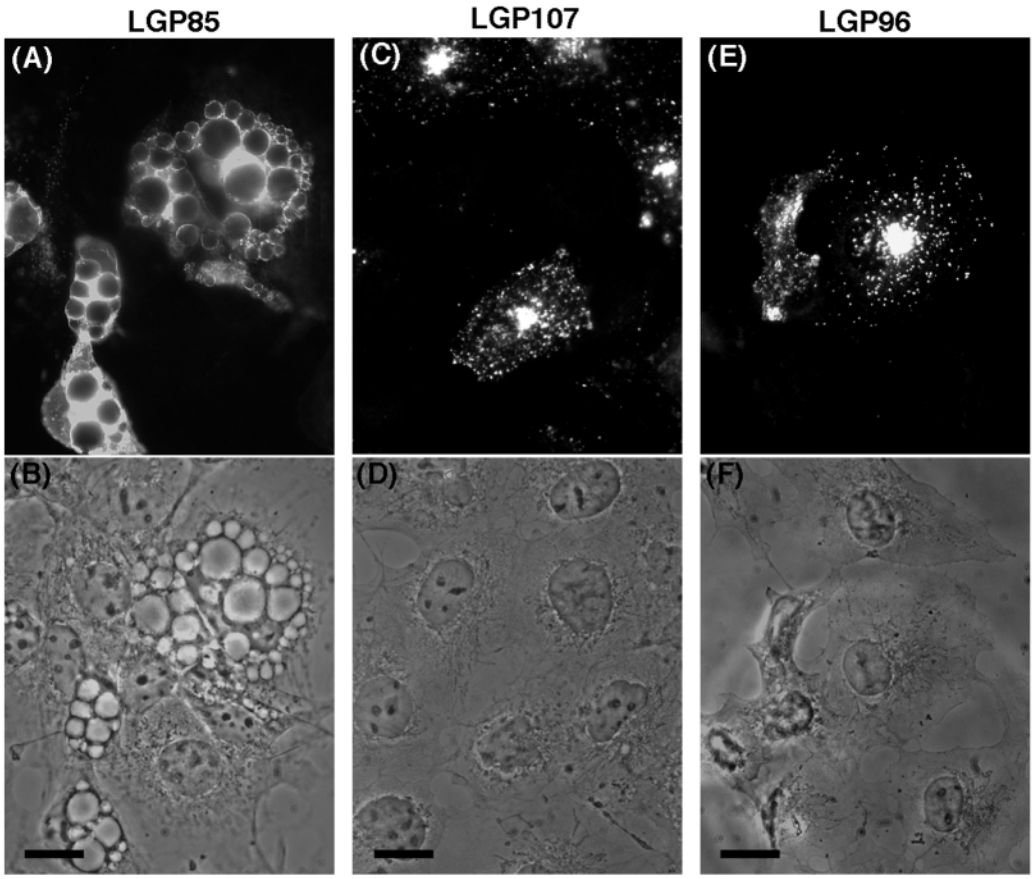

Fig. 2. The formation of large swollen vacuoles is specific to the overexpression of LGP85. COS cells were transiently transfected with LGP85 (A,B), LGP107 (C,D), or LGP96 (E,F), fixed and stained with each polyclonal antibody. Cells were visualized by immunofluorescence microscopy. Upper columns (A,C,E) show the LGP85, LGP107 and LGP96 staining, respectively, and the lower columns (B,D,F) show the corresponding phase-contrast micrographs. Bars, 20 μm.

located on one side of the nucleus and did not localize in LGP85-positive large vacuoles (Fig. 3P-R). Taken together, these results indicate that overexpression of LGP85 results in an enlargement of at least two distinct populations of endosomal compartments, early endosomes and late endosomes/lysosomes. No enlargement of TGN and Golgi compartment was observed. Therefore, we conclude that overexpression of LGP85 selectively affects endosomal compartments.

LGP85-induced large vacuoles are not derived from lysosomes, but eventually fuse with the preexisting lysosomes

We next investigated the possible involvement of lysosomes in the formation of large swollen vacuoles by overexpression of LGP85. In order to observe morphological alteration of lysosomes, cells were first incubated with Texas Red-dextran for 4 hours and chased for 20 hours to label lysosomes. Then LGP85 was transfected and the cells subjected to immunofluorescence at the indicated times after transfection. With this procedure, in untransfected cells Texas Red-dextran exhibited a small punctate staining scattered throughout the cells and in the perinuclear region, most of which colocalized with endogenous LAMP-2 (data not shown), suggesting that Texas Red-dextran-containing vesicles are mainly late endosomes/lysosomes. Interestingly, there was no significant overlap of preloaded dextran with LGP85 after 12 hours of transfection (Fig. 4A-C). However, at this time numerous large vacuoles were already visible by phase-contrast microscopy in most of the cells overexpressing LGP85, and some of them were positive for either LAMP-2 or EEA-1 (data not shown), thereby indicating that lysosomes were not involved in the initial formation of large vacuoles and overexpression of LGP85 did not cause the enlargement of lysosomes. In cells fixed 24 hours after transfection of LGP85, significant amounts of preloaded Texas Red-dextran were seen in LGP85-negative small punctate structures (Fig. 4D-F). A small number of LGP85-positive large vacuoles contained Texas Red-dextran, which was seen within or in the vicinity of the LGP85-positive large vacuoles. These results suggest that at this time most of LGP85-induced large vacuoles still have characteristics of late endosomes, but not of lysosomes. A significant colocalization between LGP85 and preloaded Texas Red-dextran in large swollen vacuoles was observed in cells 36 hours after transfection (Fig. 4G-I). Maximum overlap of the two markers was observed after 36-48 hours of expression. Taken together, these results suggest that the large vacuoles induced by overexpression of LGP85 may first arise from homotypic fusion of early and late endosomes, respectively, and the late endosome-like large vacuoles may gradually fuse with lysosomes.

Most of LGP85-induced large vacuoles are electron lucent

To characterize LGP85-induced large vacuoles in more detail, we performed immunoelectron microscopic analysis with several antibodies. As shown in Fig. 5A-C, most of LGP85-induced large vacuoles seemed empty, displaying the

appearance of swollen vacuoles, consistent with phase-contrast microscopy. The limiting membrane of these large vacuoles often appeared as double membrane (Fig. 5A,B, arrowheads). Membrane whirls and myelin figures were seen inside these large vacuoles. LAMP-2 labeling was observed mainly on the limiting membrane of the vacuoles (Fig. 5C). LBPA labeling was predominantly found associated with the internal membranes, but some labeling was closely associated with the limiting membrane (Fig. 5D, arrows). In cells expressing LGP85 at a very low level (which does not cause the formation of large vacuoles), LBPA labeling was detected in internal vesicles of MVB (Fig. 5E), similar to results reported previously (Kobayashi et al., 1998). These results suggest that an increase of vesicle fusion and/or decrease of internal vesicle invagination by overexpression of LGP85 may lead to the formation of large swollen vacuoles.

A subset of LGP85-induced large vacuoles is accessible to endocytosed tracers

To examine whether LGP85-induced large vacuoles are a compartment that is accessible to endocytic markers, COS cells expressing LGP85 were allowed to internalize either fluorescence-labeled-Tfn, -EGF, or -dextran. Tfn, which is bound to its receptor on the cell surface, is internalized via clathrin-coated vesicles, and is transferred to early endosomes. Receptor-ligand complexes are rapidly recycled back to the cell surface. Tfn was fed for 30 minutes to cells transfected with LGP85 (Fig. 6A-C). In untransfected cells, internalized Tfn predominantly distributed as small punctate structures in the perinuclear and peripheral regions, presumably representing the recycling endosome and the sorting endosome, respectively. In contrast, in cells overexpressing LGP85, Tfn was found in a subset of large swollen vacuoles positive for LGP85, which is consistent with the results obtained with double-labeling of LGP85 and EEA-1 or TfnR (Fig. 3C).

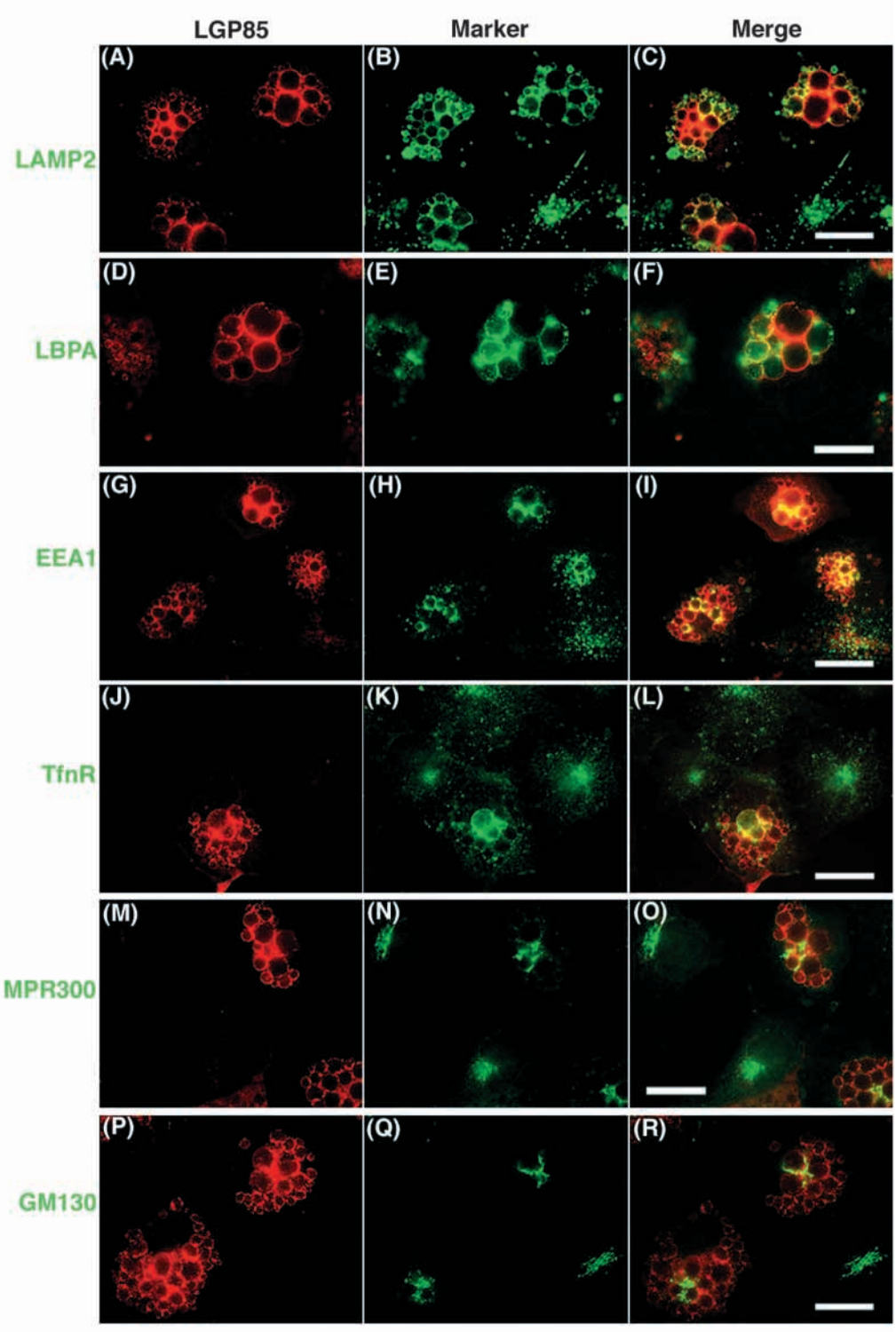

Fig. 3. Cytochemical characterization of the LGP85-induced large swollen vacuoles. COS cells were transiently transfected with LGP85, and 36 hours post-transfection were fixed and double-labeled for LGP85 (left) and LAMP-2 (B, middle), LBPA (E, middle), EEA1 (H, middle), TfnR (K, middle), MPR300 (N, middle), and GM130 (Q, middle). Cells were visualized by immunofluorescence microscopy. The right columns show the merged images of LGP85 (red) and each marker (green). Bars, 20 μm.

EGF binds to its receptor on the cell surface, and receptor-ligand complexes are internalized by clathrin-coated vesicles. Unlike Tfn, EGF and its receptor are further transported to lysosomes via endosomes and are finally degraded in lysosomes. Cells transfected with LGP85 were incubated with Texas-Red-labeled EGF for 1 hour at 4°C and chased for up to 3 hours at 37°C. After 3 hours of chase, Texas Red-EGF was seen on the limiting membrane of a subset of LGP85-positive large vacuoles (Fig. 6D-F). The same staining pattern was obtained when Texas-Red dextran was internalized for 3 hours in cells overexpressing LGP85 (Fig. 6G-I). These results indicate that only a subset of LGP85-induced vacuoles is accessible to endocytic markers.

Membrane traffic out of the early endosome is impaired by overexpression of LGP85

To characterize the nature of the LGP85-induced large vacuoles accessible for endocytic markers in more detail, we examined the distribution of these endocytic markers by double labeling with EEA1 or LAMP-2. Alexa-594-labeled Tfn was internalized for 30 minutes and the cells were fixed and labeled with either EEA1 (Fig. 7A-D) or LAMP-2 antibodies (Fig. 7M-P). In cells expressing LGP85, Alexa-594-labeled Tfn completely colocalized with EEA1 (Fig. 7A-D), but not with LAMP-2 (Fig. 7M-P), in large swollen vacuoles. EGF, which was bound to cell surface receptor for 1 hour at 4°C and chased for 3 hours at 37°C, was also seen only in EEA1-positive large swollen vacuoles (Fig. 7E-H). In untransfected cells, on the other hand, EGF was observed in LAMP-2-positive vesicles (Fig. 7Q-T), and never colocalized with EEA1 (Fig. 7E-H). The same results were obtained with Texas-red dextran (Fig. 7I-L,U-X). Taken together, these results indicate that overexpression of LGP85 causes not only an enlargement of both early endosomes and late endosomes/lysosomes but also a defect in membrane traffic from early endosomes to the late endocytic compartment. Additionally, during a subsequent 3 hours chase after withdrawal of Tfn, most of the internalized Tfn disappeared from untransfected cells, while significant amounts of the internalized Tfn were still retained in the LGP85-positive large swollen vacuoles (data not shown). Therefore, it is evident that the recycling pathway from the LGP85-induced, enlarged, early endosome-like compartment to the cell surface is also impaired.

The result that both early endosome- and late endosome-like large vacuoles appeared simultaneously leads us to speculate that the enlarged early endosomes are formed by LGP85 molecules that are delivered through an indirect route via the cell surface. If this is the case, antibodies against LGP85 added extracellularly would be taken up and retained in the early endosome-like large vacuoles induced by overexpression of LGP85. As expected, antibodies were internalized only in cells with large swollen vacuoles, in which they colocalized with EEA1, but not with LAMP-2 (Fig. 8). This is consistent with the results obtained with endocytic markers, such as Tfn, EGF and dextran. Thus, the LGP85 in early endosome-like large vacuoles may reach the endosomes through the indirect pathway of newly synthesized LGP85 via the cell surface.

Cholesterol accumulates in large swollen vacuoles induced by overexpression of LGP85

Several recent studies demonstrated that accumulation of cholesterol in late endosomes/lysosomes causes a defect of subsequent retrograde and anterograde membrane traffic (Kobayashi et al., 1999; Neufeld et al., 1999). Therefore, we examined the distribution of cellular cholesterol in LGP85-expressing cells using filipin, a specific antibiotic against free-cholesterol (Blanchette-Mackie et al., 1988). It is known that free-cholesterol predominantly distributes on the cell surface as well as the Golgi (Mukherjee and Maxfield, 2000). Indeed, in untransfected cells filipin staining was detected on the cell surface and in the perinuclear region (Fig. 9C). By contrast, overexpression of LGP85 dramatically altered filipin staining to the large vacuoles in which LGP85 was located

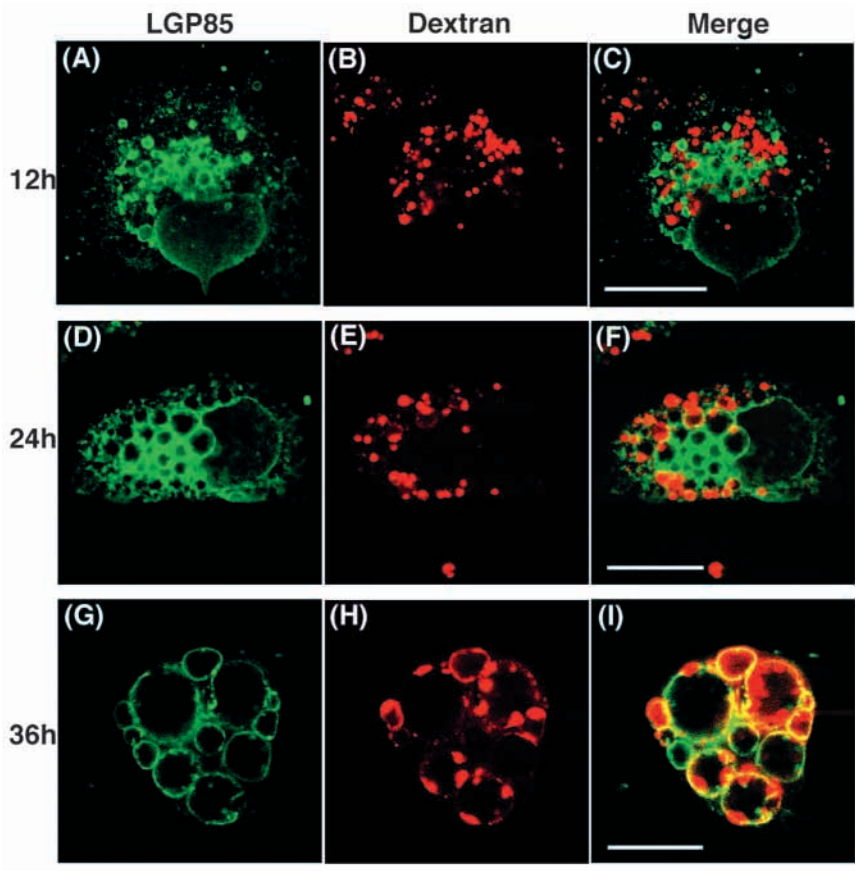

Fig. 4. Overexpression of LGP85 does not cause enlargement of lysosome, but results in formation and accumulation of the late endosome-lysosome hybrid organelle. To label lysosomes, COS cells were incubated with Texas-Red dextran for 4 hours and chased for 20 hours. After that, cells were transiently transfected with LGP85 and fixed at 12 (A-C), 24 (D-F), and 36 (G-I) hours after transfection, followed by staining for LGP85 (A,D,G, green). Cells were visualized by confocal microscopy. The right columns show the merged images of LGP85 (green) and dextran (red). Bars, 20 μm.

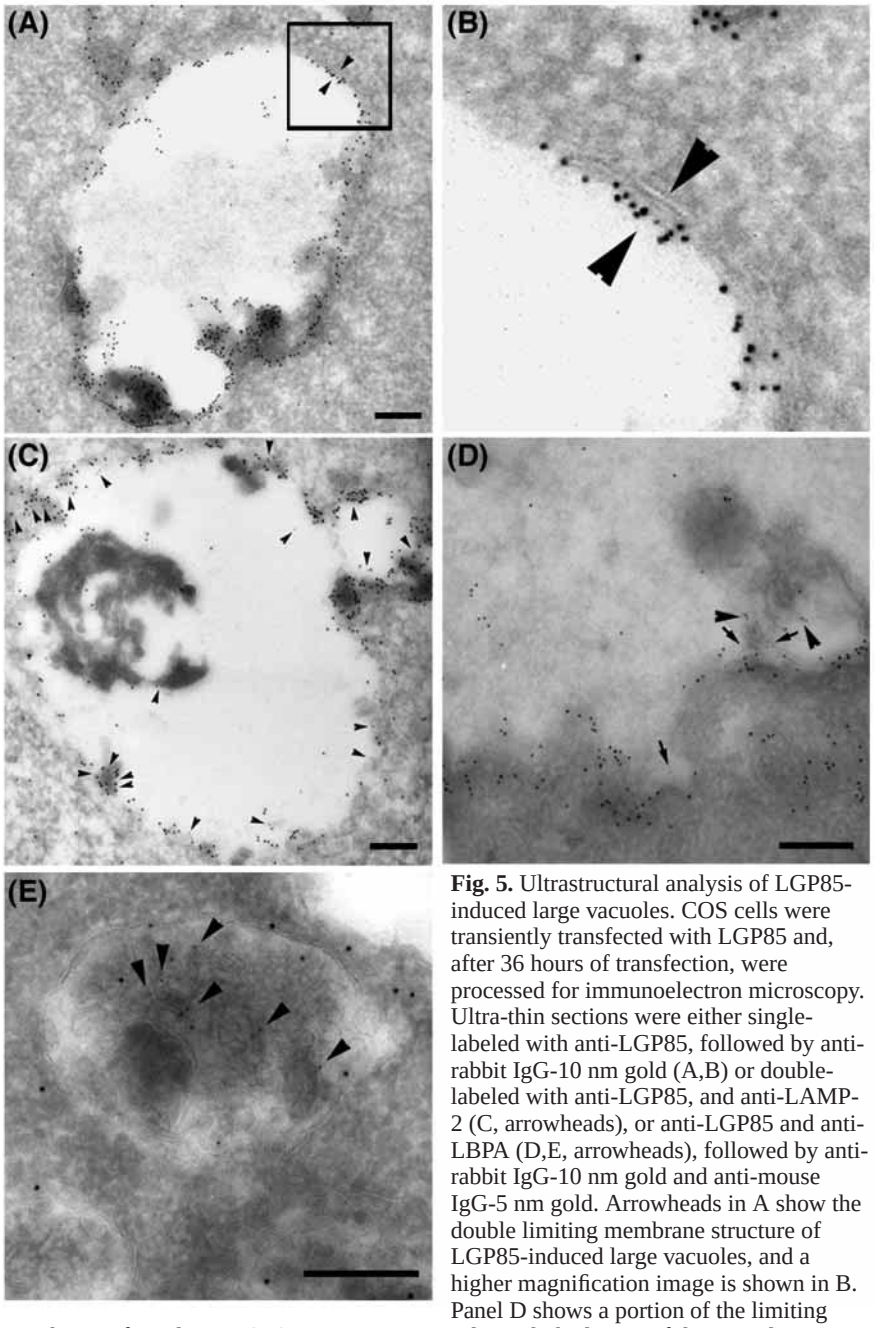

Fig. 5. Ultrastructural analysis of LGP85-induced large vacuoles. COS cells were transiently transfected with LGP85 and, after 36 hours of transfection, were processed for immunoelectron microscopy. Ultra-thin sections were either single-labeled with anti-LGP85, followed by anti-rabbit IgG-10 nm gold (A,B) or double-labeled with anti-LGP85, and anti-LAMP-2 (C, arrowheads), or anti-LGP85 and anti-LBPA (D,E, arrowheads), followed by anti-rabbit IgG-10 nm gold and anti-mouse IgG-5 nm gold. Arrowheads in A show the double limiting membrane structure of LGP85-induced large vacuoles, and a higher magnification image is shown in B. Panel D shows a portion of the limiting membrane of one large LGP85-containing vacuole, with the lumen of the vacuole occupying the upper half of the panel. Note that some LBPA labeling is seen on internal membranes closely associated with the limiting membrane of the vacuole (D, arrows). Bars, 200 nm.

(Fig. 9A-D). Neither LGP107 nor LGP96 expression caused accumulation of cholesterol in the late endosome/lysosome (data not shown).

To further examine whether the cholesterol accumulating in LGP85-positive large vacuoles is derived from lipoproteins in serum, cells were cultured for 24 hours in lipoprotein-deficient serum (LPDS) and transfected with LGP85 in a medium containing LPDS. As shown in Fig. 9E-H, large vacuoles were formed even in the presence of LPDS. There was no difference in filipin staining compared with normal medium. These results indicate that cholesterol found in LGP85-positive large vacuoles derives from either the cell surface or de novo synthesis in cells rather than from extracellular lipoproteins such as LDL. Therefore, it may be conceivable that the block of traffic out of the endosomal/lysosomal compartments in LGP85-expressing cells causes the accumulation of cholesterol in these compartments.

LGP85-induced large vacuoles are acidic

Lysosomotropic amines, such as NH_4Cl and methylamine, have been shown to cause vacuolation of acidic compartments (Ohkuma and Poole, 1981; Davis and Lyerla, 1997). The effect of these amines is due to raising the pH of intra-endosomal/lysosomal compartments by accumulation of protonated base (Ohkuma and Poole, 1981). However, the LGP85-induced large vacuoles were found to be acidic compartments as judged by staining with DAMP (data not shown), which is a basic congener of dinitropheol and accumulates in acidic organelles (Anderson et al., 1984). Specificity of the DAMP staining was demonstrated by showing that when cells were pretreated with 50 mM NH_4Cl before incubating with DAMP, the DAMP staining was no longer detected in the LGP85-induced large vacuoles nor elsewhere within the cells. In addition to the fact that the vacuolar size in NH_4Cl-treated cells is much smaller than that in LGP85-overexpressing cells (data not shown), these results imply that mechanism(s) responsible for the formation of large vacuolar compartments by overexpression of LGP85 differ from that by lysosomotropic amines.

Expression of dominant-negative Rab5b impairs the formation of large vacuoles by overexpression of LGP85

Rab GTPases are known to play key roles in regulation of membrane traffic in the endosomal/lysosomal system (Zerial and McBride, 2001). Recent studies have implicated Rab7 and Rab5a in the biogenesis of lysosomes (Bucci et al., 2000; Rosenfeld et al., 2001). Especially, expression of a GTPase-defective Rab5a (Rab5aQ79L) caused appearance of large vacuoles, which contain several lysosomal marker proteins including LAMP-1, LAMP-2 and cathepsin D (Rosenfeld et al., 2001). In an attempt to learn more about the mechanism(s) by which LGP85 induces the enlargement of endosomes/lysosomes, we examined the involvement of Rab5. Consistent with results previously reported (Rosenfeld et al., 2001), we observed that expression of FLAG-tagged GTPase-

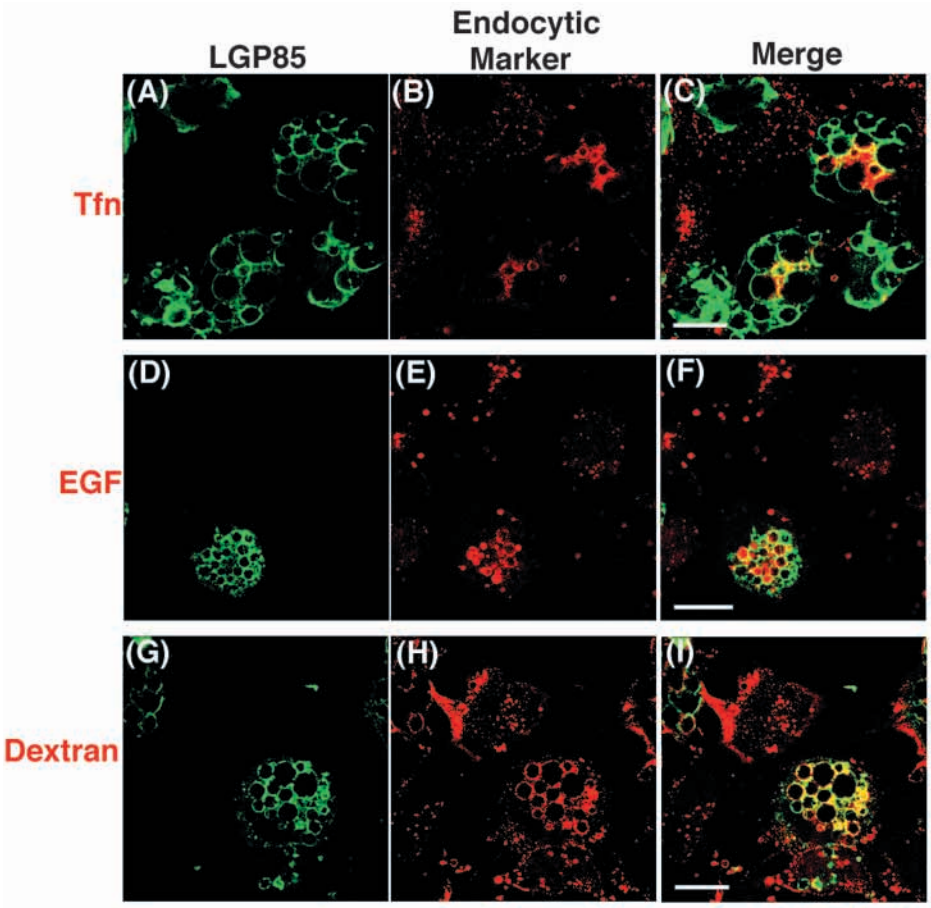

Fig. 6. A subset of LGP85-induced large swollen vacuoles is accessible to transferrin, EGF and dextran added externally. COS cells transfected with LGP85 for 36 hours were incubated with Alexa594-transferrin (red in A-C), Texas Red-EGF (red in D-F), or Texas Red-dextran (red in G-I) as described in Materials and Methods. Cells were then fixed, stained with LGP85 (green in A, D and G), and visualized by confocal microscopy. The right columns (C,F,I) show the merged images of LGP85 (green) and each endocytic marker (red). Bars, 20 μm.

defective Rab5b (Rab5bQ79L) in COS cells caused the formation of LAMP-1-positive large vacuoles (data not shown). Interestingly, co-transfection of FLAG-tagged dominant-negative Rab5b (Rab5bS34N) and LGP85 in COS cells impaired the formation of large vacuoles, leading to dispersal of LGP85-positive small vacuoles throughout the cytoplasm (Fig. 10C,D). By contrast, wild-type Rab5b (Rab5bWT) did not influence the formation of large vacuoles induced by overexpression of LGP85 (Fig. 10A,B). Rather, co-transfection of Rab5bWT and LGP85 exhibited a tendency to increase the size of and decrease the number of the vacuoles (Fig. 10A,B), which was significantly enhanced by co-transfection with Rab5bQ79L (data not shown). These results suggest the participation of a GTP-bound form of Rab5b in the formation of large vacuoles induced by overexpression of LGP85.

Discussion

In the present study, we demonstrated that overexpression of LGP85 causes the enlargement of both early endosomal and late endosomal/lysosomal compartments. This phenomenon was specific, because such large vacuoles were not seen in cells expressing vector alone or two other lysosomal membrane proteins, LGP107 (LAMP-1) or LGP96 (LAMP-2). We further demonstrated that most of these vacuoles were relatively electron lucent compartments. In addition to these morphological alterations, membrane traffic out of these compartments was also impaired by overexpression of LGP85. Since their size increased but their number decreased with increasing time of transfection (data not shown), the formation of large vacuoles induced by overexpression of LGP85 may result from (1) an increased fusion of incoming vesicles and/or homotypic fusion of endosomes and subsequent fusion of the lysosome; and (2) a defect of invagination and/or vesicle budding from the limiting membrane. It is also possible that the formation of large vacuoles is due to increased biogenesis of the limiting membrane, which could be induced by LGP85 overexpression.

Similar large vacuoles have been observed when the invariant chain (Ii) was expressed (Romagnoli et al., 1993; Stang and Bakke, 1997). Although a transient expression of the Ii in COS cells caused mainly the enlargement of the early endosome (Romagnoli et al., 1993), a stable expression of Ii in human fibroblasts induced the enlargement of early endosomes, late endosomes/prelysosomes and lysosomes (Stang and Bakke, 1997). These enlarged endosomal vacuoles were accessible for fluid phase markers, but a delay of their endosome to lysosome transport was observed. The Ii expression-induced, early endosome-like vacuoles appear to be formed by the indirect transport route of newly synthesized Ii via the cell surface (Stang and Bakke, 1997). Therefore, most of the enlarged vacuoles induced by Ii were negative for LAMP. It is conceivable that the enlarged late endosomes and lysosomes observed in cells stably expressing Ii might be a result of the maturation of the enlarged early endosomes.

By contrast, it seems unlikely that in cells overexpressing LGP85 the enlarged late endosome/lysosome-like vacuole resulted from the maturation of the early endosome-like vacuole. Such a conclusion is supported by the following results: (1) both types of vacuoles appeared simultaneously; (2) membrane traffic from the early endosome-like vacuoles to the late endosome/lysosome-like vacuole was not detected; (3) most of the enlarged vacuoles were positive for LAMP-2. Rather, as antibodies against LGP85 fed extracellularly were internalized and colocalized with EEA1-positive, but not with LAMP-2-positive large swollen vacuoles, the early endosome-like vacuoles observed in COS cells may arise due to excessive supply of LGP85 from the cell surface to early endosomes,

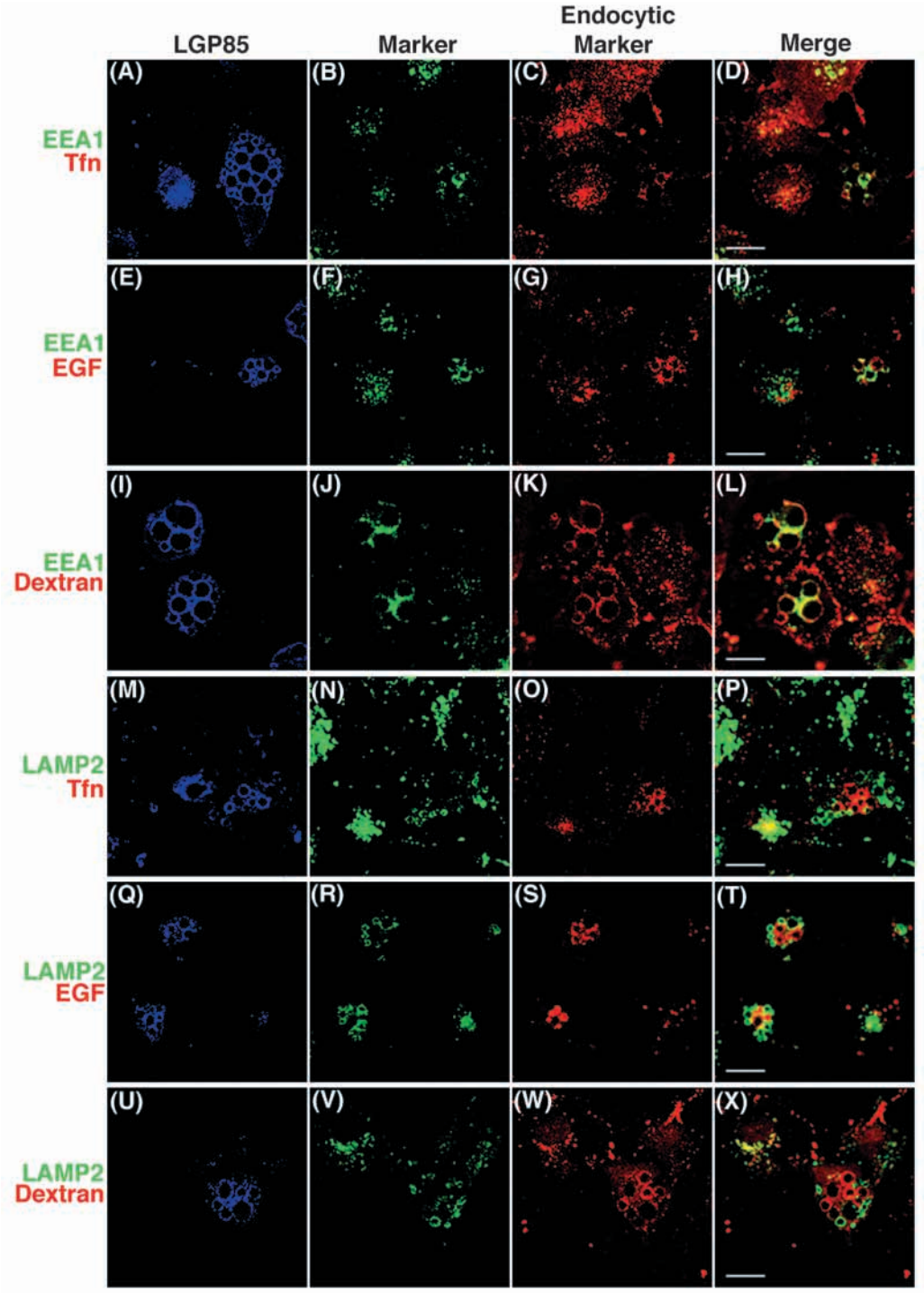

Fig. 7. Overexpression of LGP85 causes impairment of membrane traffic from early endosomes to late endosomes/lysosomes. COS cells transfected with LGP85 for 36 hours were incubated with Alexa594-transferrin (red in C and O), Texas Red-EGF (red in G and S), or Texas Red-dextran (red in K and W) as described in Materials and Methods. Cells were then fixed, stained with LGP85 (blue in left columns) and either EEA1 (green in B, F and J) or LAMP-2 (green in N, R and V), and visualized by confocal microscopy. The right columns (D, H, L, P, T and X) show the merged images of EEA1 (green in D, H and L) or LAMP-2 (green in P, T and X) and each endocytic marker (red). Bars, 20 μm.

which may be a consequence of missorting from the TGN by overexpression. It is also possible that increased fusion of LGP85-containing TGN-derived vesicles with pre-existing early endosomes might facilitate the formation of these large vacuoles.

Overexpression of LGP85 did not cause an enlargement of lysosomes that were labeled by preloading dextran before transfection of LGP85. Indeed, preloaded dextran did not colocalize with LGP85-induced, LBPA- and LAMP-2-positive, large vacuoles already formed at 12 hours post-transfection, indicating that there is no direct involvement of lysosomes in the initial formation of large vacuoles. Our results further showed that lysosomes fuse with the preformed enlarged LBPA and LAMP-2-positive vacuoles. These results may reflect that LGP85-induced large vacuoles are formed first by homotypic fusion of late endosomes, and gradually tend to fuse with lysosomes. Thus, LGP85-induced late endosome/lysosome-like vacuoles seem to resemble the hybrid organelle, which is transiently formed by direct fusion between the late endosome and the lysosome (Bright et al., 1997).

The late endosome-lysosome hybrid organelle was originally identified in cells treated with the phosphoinositide

(PI) 3-kinase inhibitor, wortmannin (Reaves et al., 1996). Wortmannin causes enlargement of late endocytic/pre-lysosomal compartments, but not of lysosomes containing acid hydrolases, as well as redistribution of lysosomal membrane proteins (lgp120 and lgp110) to enlarged late endosomes from lysosomes. However, prolonged treatment of cells with wortmannin (5 hours) eventually resulted in direct fusion between swollen endosomes and lysosomes and concomitant decrease of dense core lysosomes (Bright et al., 1997). Thus, it has been suggested that wortmannin causes an accumulation of late endosome-lysosome hybrid organelles as a result of the inhibition of membrane traffic out of the hybrid organelles (e.g. inhibition of reformation of lysosomes). Similarly, we could not detect reformation of lysosomes in cells overexpressing

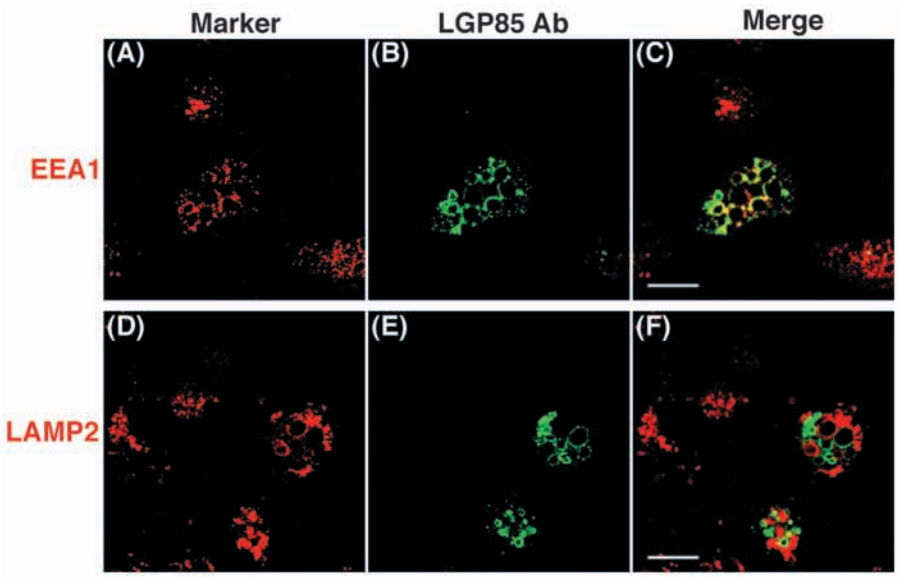

Fig. 8. Anti-LGP85 antibodies are internalized to early endosome-like large vacuoles. COS cells transfected with LGP85 for 36 hours were incubated for 1 hour with rabbit polyclonal antibodies against LGP85 at 4°C and chased for 3 hours at 37°C. Cells were then fixed, permeabilized, and incubated with a mouse monoclonal antibody to either EEA1 (A) or LAMP-2 (D). Localization of internalized and bound antibodies revealed by Alexa488-conjugated goat anti-rabbit IgG (green in B and E) and Alexa594-conjugated goat anti-mouse antibody (red in A and D), respectively, was visualized by confocal microscopy. The right columns (C,F) show the merged images of internalized antibodies (green in B and E) and EEA1 (red in A) or LAMP-2 (red in D). Bars, 20 μm.

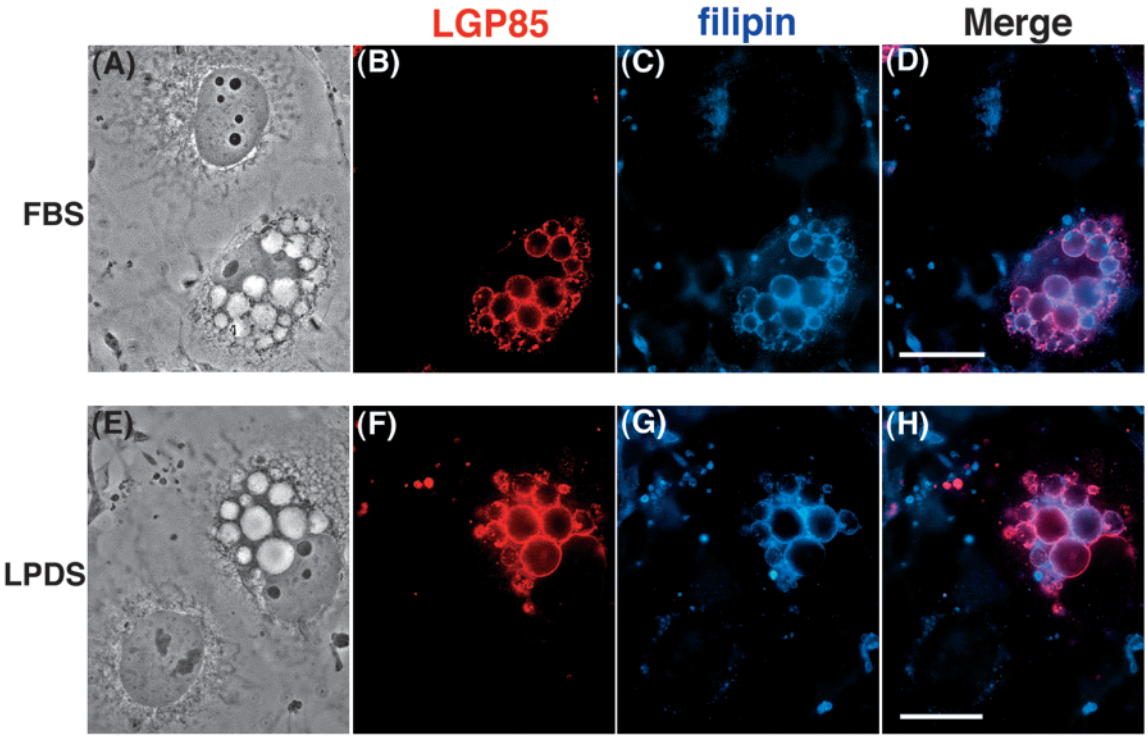

Fig. 9. Accumulation of cholesterol in LGP85-induced large vacuolar compartments. COS cell cultured in DMEM supplemented with 10% FBS were transiently transfected with LGP85 and fixed after 36 hours after transfection. Cells were immunostained for LGP85 (B) and cytochemically stained with filipin (C) to detect cholesterol. To examine further whether cholesterol accumulated in the LGP85-induced large swollen vacuoles is derived from one containing into cellular membranes or into LDL, COS cells cultured in DMEM supplemented with 10% LPDS instead of 10% FBS for 2 days were transiently transfected with LGP85, fixed at 36 hours after transfection, and stained for LGP85 (F) and cholesterol (G). The right columns (D,H) show the merged images of LGP85 (red) and cholesterol (blue), and the left columns (A,E) show the corresponding phase-contrast micrographs. Bars, 20 μm.

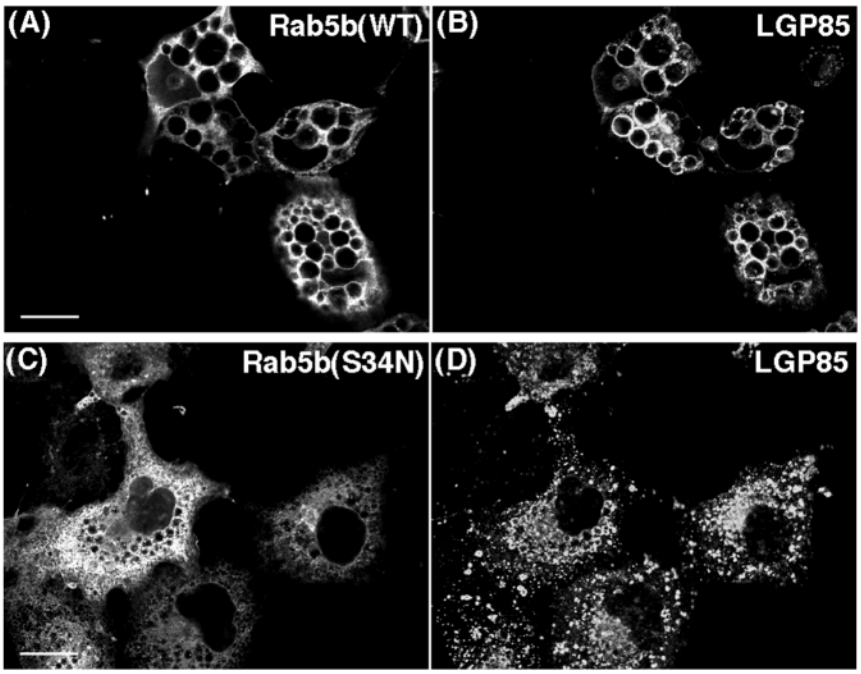

Fig. 10. The dominant-negative form of Rab5b inhibits the formation of LGP85-induced large vacuoles. COS cells were transiently co-transfected with LGP85 and FLAG-tagged wild-type Rab5b or FLAG-tagged Rab5bS34N, and after 36 hours post-transfection fixed and incubated with a rabbit polyclonal antibody to LGP85 (B,D) and a mouse monoclonal antibody to FLAG for labeling of wild-type and mutant Rab5b (A,C). Cells were visualized by confocal microscopy. Bars, 20 μm.

LGP85. We conclude that accumulation of LGP85 in the endosomal compartment could interfere with vesicle budding without inhibiting the vesicle fusion event, leading to the enlargement of this compartment.

The block of traffic out of LGP85-induced large vacuoles may correlate with the accumulation of cholesterol in these structures. There is considerable evidence to support a role for cholesterol as an important molecule in the context of the intracellular membrane traffic: accumulation of cholesterol into late endosomes/lysosomes by administrating antibodies to LBPA (Kobayashi et al., 1999) and by mutating the Niemann-Pick type C disease gene (Neufeld et al., 1999) leads to impairment of membrane traffic out of these compartments. Expression of an ATPase-defective form of human Vps4p, which is the mammalian homologue of yeast Vps4p required for endosomal trafficking (Babst et al., 1998), has also been known to cause not only enlargement of multiple endosomal compartments and defects in postendosomal sorting, but also accumulation of cholesterol in these compartments (Bishop and Woodman, 2000), consistent with phenotypes induced by overexpression of LGP85. Authors have pointed out that the phenotype observed in the cells expressing mutant human Vps4p is strikingly similar to that induced by wortmannin.

In addition to the inhibition of reformation of lysosomes, wortmannin has been proposed to induce enlargement of late endosomes and lysosomes by inhibiting exit of proteins such as MPR (Kundra and Kornfeld, 1998) or invagination and/or pinching off of intraluminal vesicles (Fernandez-Borja et al., 1999; Futter et al., 2001). It was indicated that class III PI 3-kinase, the human VPS34, is indeed involved in inward vesiculation, suggesting a role for PtdIns3*P* in the regulation of MVB morphogenesis. Besides the early requirement for PtdIns3*P* as a regulator of vesicle docking/fusion at the endosome through the recruitment/activation of components in the PI 3-kinase signaling cascade (Lemmon and Traub, 2000; Odorizzi et al., 2000; Simonsen et al., 2001), the generation of PtdIns(3,5)P_2 from PtdIns3*P* is also required for the vesicle invagination and/or cargo selection within the MVBs (Odorizzi et al., 1998). Indeed, in yeast, the loss of Fab1p, a protein previously identified in yeast as PtdIns3*P* 5-kinase, which phosphorylates PtdIns3*P* to produce PtdIns(3,5)P_2, causes an abnormally large swollen vacuole containing far fewer internal vesicles (Gary et al., 1998). It has recently been demonstrated that PIKfyve, a mammalian ortholog of Fab1p (Shisheva et al., 1999; McEwen et al., 1999), localizes on MVBs (Shisheva et al., 2001), and transient expression of a kinase-deficient point mutant of PIKfyve (PIKfyve^{K1831E}) in COS cells results in multiple large swollen vacuoles that originate from late endosomes (Ikonomov et al., 2001). PIKfyve^{K1831E}-induced vacuoles have diameters of 5-10 μm, and increase in size and decrease in number with increasing time post-transfection. Such dominant phenotypes observed with PIKfyve^{K1831E} are very similar to those induced by overexpression of LGP85 presented in this study, thereby suggesting that a common mechanism might be involved. We observed that the LGP85-induced large vacuoles were morphologically indistinguishable from those induced by wortmannin treatment (data not shown). Immunoelectron microscopy further showed that most, but not all, of LGP85-induced large vacuoles have few internal membranous structures. Thus it is possible that overexpression of LGP85 impaired the formation of MVB. One possibility is that overexpression of LGP85 may cause the formation of large swollen vacuoles by decreasing internal vesicle formation from the limiting membrane, possibly by inhibiting the membrane recruitment of PI3-kinase and/or PIKfyve.

Nevertheless, our results that EEA1 is associated with the membrane of the LGP85-induced large vacuoles suggests that PtdIns3*P* is present in these vacuoles. EEA1 binds to endosomal membranes through interaction with PtdIns3*P* and is released from the membrane by wortmannin treatment (Simonsen et al., 1998). Therefore, overexpression of LGP85 may not exert the generation of PtdIns3*P* itself. The efficient recruitment of EEA1 on early endosomes requires the concomitant presence of Rab5 and PtdIns3*P* (Christoforidis et al., 1999a). Furthermore, the GTP-bound form of Rab5 specifically interacts with class I and III PI 3-kinases (Christoforides et al., 1999b). However, there is evidence that the presence of excess activated Rab5 on the membrane can compensate the membrane association of EEA1 for the lack of PtdIns3*P* in the presence of wortmannin (Simonsen et al., 1998). Recent studies further indicate that the enlarged endosomes induced by wortmannin are the same endosomes

induced by Rab5Q79L, and suggest that wortmannin can stimulate Rab5 activity by blocking the interaction between Rab5 and p120 Ras GAP, a Rab5 GTPase-activating protein, rather than inhibiting PI 3-kinase (Chen and Wang, 2001). We observed a dot-like staining pattern of EEA1 on the LGP85-induced large vacuolar membranes. This staining pattern is reminiscent of that observed in enlarged early endosomes by expression of Rab5Q79L, representing membrane microdomains, which function as tethering platforms for incoming vesicles (McBride et al., 1999). We further found that expression of the dominant-negative mutant of Rab5b abolishes the formation of LGP85-induced large vacuoles. These findings led to the suggestion that enhanced Rab5b activation in the cells overexpressing LGP85 may facilitate the formation of large vacuoles through stimulation of endosome-endosome fusion. It is interesting to note that expression of the dominant-negative mutant of Rab5b impaired the formation of not only early endosome-like but also late endosome/lysosome-like large vacuoles induced by overexpression of LGP85, because Rab5 is known to participate in homotypic fusion of early endosomes (Gorvel et al., 1991; Bucci et al., 1992). It has recently been reported, however, that expression of Rab5aQ79L causes redistribution of lysosomal proteins into Rab5aQ79L-positive enlarged vacuoles, in which Tfn is rapidly internalized (Rosenfeld et al., 2001). Thus, together with our findings, it is conceivable that Rab5 plays a crucial role in the biogenesis of lysosomes, which may need appropriate membrane traffic from, or maturation of, early endosomes. In contrast to LGP85, expression of Rab5bQ79L did not cause accumulation of cholesterol in the enlarged endosomal compartments (data not shown); therefore, additional molecule(s) could be involved in the formation of LGP85-induced large vacuoles. In fact, evidence that at least 20 proteins directly or indirectly interact with the GTP-bound form of Rab5 (Christoforidis et al., 1999) suggests that the machinery downstream of this GTPase is extremely complex.

At present we have no direct evidence for the molecular mechanism that leads to the block in transport and enlargement of endosomal/lysosomal compartments by overexpression of LGP85. However, it is likely that the cytoplasmic tail and/or the transmembrane domain of LGP85 might be involved. So far, there are no proteins that are known to interact with the transmembrane domain, while it has been demonstrated that AP-3 can interact with the cytoplasmic tail of LGP85 (Höning et al., 1998). The identification of binding partners for LGP85 by two-hybrid or co-immunoprecipitation studies should help to shed light on the molecular mechanism(s) by which LGP85 regulates the biogenesis of lysosomes.

We conclude that LGP85 might control the balance between vesicle invagination versus vesicle budding from the limiting membrane of endosomal compartments. Since the formation of large vacuoles was dependent on the expression level of LGP85, it is possible that overexpression of LGP85 may cause a dispersal of the budding machinery, which might be due to impaired recruitment of a cytoplasmic factor involved in vesicular fission and/or fusion. LGP85 may play a key role in regulating the late endosomal/lysosomal compartment, possibly as one of the components of molecular machinery necessary for the intercompartmental membrane trafficking along the endocytic pathway rather than as a constituent of late endosomes and lysosomes. To our knowledge, this is the first report of a role for LGP85 in the biogenesis and maintenance of endosomes/lysosomes.

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References

Anderson, R. G. W., Falck, J. R., Goldstein, J. L. and Brown, M. S. (1984). Visualization of acidic organelles in intact cells by electron microscopy. *Proc. Natl. Acad. Sci. USA* **81**, 4838-4842.

Babst, M., Wendland, B., Estepa, E. J. and Emr, S. D. (1998). The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required fro normal endosome function. *EMBO J*. **17**, 2982-2993.

Barriocanal, J., Bonifacino, J., Yuan, L. and Sandoval, I. V. (1986). Biosynthesis, glycosylation, movement through the Golgi system, and transport to lysosomes by an *N*-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. *J. Biol. Chem*. **261**, 16755-16763.

Bishop, N. and Woodman, P. (2000). ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking. *Mol. Biol. Cell* **11**, 227-239.

Blanchette-Mackie, E. J., Dwyer, N. K., Amende, L. M., Kruth, H. S., Butler, J. D., Sokol, J., Comly, M. E., Vanier, M. T., August, J. T., Brady, R. O. and Pentchev, P. G. (1988) Type-C Niemann-Pick disease: low density lipoprotein uptake is associated with premature cholesterol accumulation in the Golgi complex and excessive cholesterol storage in lysosomes. *Proc. Natl. Acad. Sci. USA* **85**, 8022-8026.

Bock, J. B., Klumperman, J., Davanger, S. and Scheller, R. H. (1997). Syntaxin 6 functions in trans-Golgi network vesicle trafficking. *Mol. Biol. Cell* **8**, 1261-1271.

Bright, N. A., Reaves, B. J., Mullock, B. M. and Luzio, J. P. (1997). Dense core lysosomes can fuse with late endosomes and are re-formed from the resultant hybrid organelles. *J. Cell Sci*. **110**, 2027-2040.

Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B. and Zerial, M. (1992). The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell* **70**, 715-728.

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J. and van Deurs, B. (2000). Rab7: a key to lysosome biogenesis. *Mol. Biol. Cell* **11**, 467-480.

Chen, X. and Wang, Z. (2001). Regulation of epidermal growth factor receptor endocytosis by wortmannin through activation of Rab5 rather than inhibition of phosphatidylinositol 3-kinase. *EMBO Rep.* **2**, 842-849.

Christoforidis, S., McBride, H. M., Burgoyne, R. D. and Zerial, M. (1999a). The Rab5 effector EEA1 is a core component of endosome docking. *Nature* **397**, 621-625.

Christoforidis, S., Miaczynska, M., Ashman, K., Wilm, M., Zhao, L., Yip, S. C., Waterfield, M. D., Backer, J. M. and Zerial, M. (1999b). Phosphatidylinositol-3-OH kinases are Rab5 effector. *Nat. Cell Biol*. **1**, 249-252.

Davis, S. G. and Lyerla, T. A. (1997). The effect of lysosomotropic amines on *beige* mouse cells. *Exp. Cell Res*. **237**, 242-246.

Dell'Angelica, E. C., Shotelersuk, V., Anguilar, R. C., Gahl, W. A. and Bonifacino, J. S. (1999). Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. *Mol. Cell* **3**, 11-21.

de Duve, C. (1983). Lysosomes revisited. *Eur. J. Biochem*. **137**, 391-397.

Felder, S., Miller, K., Moehren, G., Ullich, A., Schlessinger, J. and Hopkins, C. R. (1990). Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. *Cell* **61**, 623-634.

Fernandez-Borja, M., Wubbolts, R., Calafat, J., Janssen, H., Divecha, N., Dusseljee, S. and Neefjes, J. (1999). Multivesicular body morphogenesis requires phosphatidyl-inositol 3-kinase activity. *Curr. Biol*. **9**, 55-58.

Fujita, H., Ezaki, J., Noguchi, Y., Kono, A., Himeno, M. and Kato, K. (1991). Isolation and sequencing of a cDNA clone encoding 85kDa sialoglycoprotein in rat liver lysosomal membranes. *Biochem. Biophys. Res. Commun*. **178**, 444-452.

Fujita, H., Takata, K., Kono, A., Tanaka, Y., Takahashi, K. Himeno, M. and Kato, K. (1992). Isolation and sequencing of a cDNA clone encoding the 85kDa human lysosomal sialoglycoprotein (hLGP85) in human metastatic pancreas islet tumor cells. *Biochem. Biophys. Res. Commun*. **184**, 604-611.

Fukuda, M. (1991). Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *J. Biol. Chem*. **266**, 21327-21330.

Furuno, K., Ishikawa, T., Akasaki, K., Yano, S., Tanaka, Y., Yamaguchi, Y., Tsuji, H., Himeno, M. and Kato, K. (1989a). Morphological localization of a major lysosomal membrane glycoprotein in the endocytic membrane system. *J. Biochem. (Tokyo)* **106**, 708-716.

Furuno, K., Yano, S., Akasaki, K., Tanaka, Y., Yamaguchi, Y., Tsuji, H., Himeno, M. and Kato, K. (1989b). Biochemical analysis of the movement of a major lysosomal membrane glycoprotein in the endocytic membrane system. *J. Biochem. (Tokyo)* **106**, 717-722.

Futter, C. E., Pearse, A., Hewlett, L. J. and Hopkins, C. R. (1996). Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. *J. Cell Biol*. **132**, 1011-1023.

Futter, C. E., Collinson, L. M., Backer, J. M. and Hopkins, C. R. (2001). Human VPS34 is rewuired for intenal vesicle formation within multivesicular endosomes. *J. Cell Biol*. **155**, 1251-1263.

Gary, J. D., Wurmser, A. E., Bonangelino, C. J., Weisman, L. S. and Emr, S. D. (1998). Fab1p is essential for Ptdlns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. *J. Cell Biol*. **143**, 65-79.

Gorvel, J. P., Chavrier, P., Zerial, M. and Gruenberg, J. (1991). Rab5 controls early endosome fusion in vitro. *Cell* **64**, 915-925.

Gough, N. R. and Fambrough, D. M. (1997). Different steady state subcellular distributions of the three splice variants of lysosome-associated membrane protein LAMP-2 are determined largely by the COOH-terminal amino acid residue. *J. Cell Biol*. **137**, 1161-1169.

Gough, N. R., Zweifel, M. E., Martinez-Augustin, O., Augilar, R. C., Bonifacino, J. S. and Fambrough, D. M. (1999). Utilization of the indirect lysosome targeting pathway by lysosome-associated membrane proteins (LAMPs) is influenced largely by the C-terminal residue of their GYXXϕ targeting signals. *J. Cell Sci*. **112**, 4257-4269.

Griffith, G., Hoflack, B., Simons, K., Mellman, I. and Kornfeld, S. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* **52**, 329-341.

Gruenberg, J. and Maxfield, F. R. (1995). Membrane transport in the endocytic pathway. *Curr. Opin. Cell Biol*. **7**, 552-563.

Guarnieri, F. G., Arterburn, L. M., Penno, M. B., Cha, Y. and August, J. T. (1993). The motif Tyr-X-X-hydrophobic residue mediates lysosomal membrane targeting of lysosome-associated membrane protein 1. *J. Biol. Chem*. **268**, 1941-1946.

Harter, C. and Mellman, I. (1992). Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane. *J. Cell Biol*. **117**, 311-325.

Himeno, M., Noguchi, Y., Sasaki, H., Tanaka, Y., Furuno, K., Kono, A., Sakaki, Y. and Kato, K. (1989). Isolation and sequencing of a cDNA clone encoding 107kDa sialoglycoprotein in rat liver lysosomal membranes. *FEBS Lett.* **244**, 351-356.

Höning, S. and Hunziker, W. (1995). Cytoplasmic determinants involved in direct lysosomal sorting, endocytosis, and basolateral targeting of rat lgp120 (lamp-1) in MDCK cells. *J. Cell Biol*. **128**, 321-332.

Höning, S., Sandval, I. V. and von Figura, K. (1998). A di-leucine-based motif in the cytoplasmic tail of LIMP II and tyrosinase mediates selective binding of AP-3. *EMBO J*. **17**, 1304-1314.

Hunziker, W. and Geuze, H. J. (1996). Intracellular trafficking of lysosomal membrane proteins. *BioEssays* **18**, 379-389.

Ikonomov, O. C., Sbrissa, D. and Shisheva, A. (2001). Mammalian cell morphology and endocytic membrane homeostasis require enzymatically active phosphoinositide 5-kinase PIKfyve. *J. Biol. Chem*. **276**, 26141-26147.

Kirchhausen, T. (1999). Adaptors for clathrin-mediated traffic. *Annu. Rev. Cell Dev. Biol*. **15**, 705-732.

Kobayashi, T., Stang, E., Fang, K. S., de Moerloose, P., Parton, R. G. and Gruenberg, J. (1998). A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. *Nature* **392**, 193-197.

Kobayashi, T., Beuchat, M.-H., Lindsay, M., Frias, S., Palmiter, R. D., Sakuraba, H., Parton, R. G. and Gruenberg, J. (1999). Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. *Nat. Cell Biol*. **1**, 113-118.

Kornfeld, S. (1992). Structure and function of the mannose 6-phosphate insulin-like growth factor II receptors. *Annu. Rev. Biochem*. **61**, 307-330.

Kornfeld, S. and Mellman, I. (1989). The biogenesis of lysosomes. *Annu. Rev. Cell Biol*. **5**, 483-525.

Kundra, R. and Kornfeld, S. (1998). Wortmannin retards the movement of the mannose 6-phosphate/insulin-like growth factor II receptor and its ligand out of endosomes. *J. Biol. Chem*. **273**, 3848-3853.

Kurosu, H. and Katada, T. (2001). Association of phosphatidylinositol 3-kinase composed of p110β-catalytic and p85-regulatory subunits with the small GTPase Rab5. *J. Biochem. (Tokyo)* **130**, 73-78.

Le Borgne, R., Alconada, A., Bauer, U. and Hoflack, B. (1998). The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins. *J. Biol. Chem*. **273**, 29451-29461.

Lemmon, S. K. and Traub, L. M. (2000). Sorting in the endosomal system in yeast and animal cells. *Curr. Opin. Cell Biol*. **12**, 457-466.

Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L. and Klausner, R. D. (1991). Brefeldin A's effects on endosomes, lysosomes and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. *Cell* **67**, 601-616.

Luzio, J., Rous, B. A., Bright, N. A., Pryor, P. R., Mullock, B. M. and Piper, R. C. (2000). Lysosome-endosome fusion and lysosome biogenesis. *J. Cell Sci*. **113**, 1515-1524.

MacDonald, R. G., Pfeffer, S. R., Coussens, L., Tepper, M. A., Brocklebank, C. M., Mole, J. E., Anderson, J. K., Chen, E., Czech, M. P. and Ullrich, A. (1988). A single receptor binds both insulin-like growth factor II and mannose-6-phosphate. *Science* **239**, 1134-1137.

McBride, H. M., Rybin, V., Murphy, C., Giner, A., Teasdale, R. and Zerial, M. (1999). Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntax in 13. *Cell* **98**, 377-386.

McEwen, R. K., Dove, S. K., Cooke, F. T., Painter, G. F., Holmes, A. B., Shisheva, A., Ohya, Y., Parker, P. J. and Michell, R. H. (1999). Complementation analysis in PtdInsP Kinase-deficient yeast mutants demonstrates that Schizosaccharomyces pombe and murine Fab1p homologues are phosphatidylinositol 3-phosphate 5-kinases. *J. Biol. Chem*. **274**, 33905-33912.

Mellman, I. (1996). Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol*. **12**, 575-625.

Mu, F. T., Callaghan, J. M., Steele-Mortimer, O., Stenmark, H., Parton, R. G., Campbell, P. L., McCluskey, J., Yeo, J. P., Tock, E. P. and Toh, B. H. (1995). EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif. *J. Biol. Chem*. **270**, 13503-13511.

Mukherjee, S. and Maxfield, F. R. (2000). Role of membrane organization and membrane domains in endocytic lipid trafficking. *Traffic* **1**, 203-211.

Mullock, B. M., Bright, N. A., Fearon, C. W., Gray, S. R. and Luzio, J. P. (1998). Fusion of lysosomes with late endosomes produces a hybrid organelle of intermediate density and is NSF dependent. *J. Cell Biol*. **140**, 591-601.

Nakamura, N., Raboville, C., Watson, R., Nilsson, T., Hui, P., Slusarewicz, N., Kreis, T. H. and Warren, G. (1995). Characterization of a *cis*-Golgi matrix protein, GM130. *J. Cell Biol*. **131**, 1715-1726.

Neufeld, E. B., Wastney, M., Patel, S., Suresh, S., Cooney, A. M., Dwyer, N. K., Roff, C. F., Ohno, K., Morris, J. A., Carstea, E. D. et al. (1999). The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal acrgo. *J. Biol. Chem*. **274**, 9627-9635.

Noguchi, Y., Himeno, M., Sasaki, H., Tanaka, Y., Kono, A., Sakaki, Y. and Kato, K. (1989). Isolation and sequencing of a cDNA clone encoding 96kDa sialoglycoprotein in rat liver lysosomal membranes. *Biochem. Biophys. Res. Commun*. **164**, 1113-1120.

Odorizzi, G., Babst, M. and Emr, S. D. (1998). Fab1p Ptdlns(3)P 5-kinase function essential for protein sorting in the multivesicular body. *Cell* **95**, 847-858.

Odorizzi, G., Babst, M. and Emr, S. D. (2000). Phosphoinositide signaling and the regulation of membrane trafficking in yeast. *Trends Biochem. Sci*. **25**, 229-235.

Ogata, S. and Fukuda, M. (1994). Lysosomal targeting of Limp II membrane glycoprotein requires a novel Leu-Ile motif at a particular position in its cytoplasmic tail. *J. Biol. Chem*. **269**, 5210-5217.

Ohkuma, S. and Poole, B. (1981). Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances. *J. Cell Biol*. **90**, 656-664.

Ohsumi, Y., Ishikawa, T. and Kato, K. (1983). A rapid and simplified method for the preparation of lysosomal membranes from rat liver. *J. Biochem. (Tokyo)* **93**, 547-556.

Okazaki, I., Himeno, M., Ishikawa, T. and Kato, K. (1992). Purification and characterization of an 85kDa sialoglycoprotein in rat liver lysosomal membranes. *J. Biochem. (Tokyo)* **111**, 763-769.

Peters, C. and von Figura, K. (1994). Biosynthesis of lysosomal membranes. *FEBS Lett.* **346**, 108-114.

Reaves, B. J., Bright, N. A., Mullock, B. M. and Luzio, J. P. (1996). The effect of wortmannin on the localisation of lysosomal type I integral membrane glycoproteins suggests a role for phosphoinositide 3-kinase activity in regulating membrane traffic late in the endocytic pathway. *J. Cell Sci*. **109**, 749-762.

Robinson, M. S. and Bonifacino, J. S. (2001). Adaptor-related proteins. *Curr. Opin. Cell Biol*. **13**, 444-453.

Romagnoli, P., Layet, C., Yewdell, J., Bakke, O. and Germain, R. N. (1993). Relationship between invariant chain expression and major histocompatibility complex class II transport into early and late endocytic compartments. *J. Exp. Med*. **177**, 583-596.

Rosenfeld, J. L., Moore, R. H., Zimmer, K.-P., Alpizar-Foster, E., Dai, W., Zarka, M. N. and Knoll, B. J. (2001). Lysosome proteins are redistributed during expression of a GTP-hydrolysis-defective rab5a. *J. Cell Sci*. **114**, 4499-4508.

Sandoval, I. V., Arredondo, J. J., Alcalde, J., Gonzalez-Noriega, A., Vandekerckhove, J., Jimenez, M. A. and Rico, M. (1994). The residues Leu(Ile)^{475}-Ile(Leu, Val, Ala)^{476}, contained in the extended carboxyl cytoplasmic tail, are critical for targeting of the resident lysosomal membrane protein LIMP II to lysosomes. *J. Biol. Chem*. **269**, 6622-6631.

Shisheva, A., Sbrissa, D. and Ikonomov, O. (1999). PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. *Mol. Cell. Biol*. **19**, 623-634.

Shisheva, A., Rusin, B., Ikonomov, O. C., DeMarco, C. and Sbrissa, D. (2001). Localization and insulin-regulated relocation of phosphoinositide 5-kinase PIKfyve in 3T3-L1 adipocytes. *J. Biol. Chem*. **276**, 11859-11869.

Simonsen, A., Lippe, R., Christoforidis, S., Gauller, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M. and Stenmark, H. (1998). EEA1 links PI(3)k function to Rab5 regulation of endosome fusion. *Nature* **394**, 494-498.

Simonsen, A., Wurmser, A. E., Emr, S. D. and Stenmark, H. (2001). The role of phosphoinositides in membrane transport. *Cuur. Opin. Cell Biol*. **13**, 485-492.

Stang, E. and Bakke, O. (1997). MHC class II-associated invariant chain-induced enlarged endosomal structures: A morphological study. *Exp. Cell Res*., **235**, 79-92.

Stoorvogel, W., Strous, G. J., Geuze, H. J., Oorschot, V. and Schwartz, A. L. (1991). Late endosomes derive from early endosomes by maturation. *Cell* **65**, 417-427.

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.

van Deurs, B., Holm, P. K., Kayser, L., Sandvig, K. and Hansen, S. H. (1993). Multivesiclular bodies in Hep-2 cells are maturing endosomes. *Eur. J. Cell Biol*. **61**, 208-224.

Vega, M. A., Segui-Real, B., Alcalde-Garcia, J., Cales, C., Rodriguez, F., Vanderkerckhove, J. and Sandoval, I. V. (1991). Cloning, sequencing, and expression of a cDNA encoding rat LIMP II, a novel 74-kDa lysosomal membrane protein related to the surface adhesion protein CD36. *J. Biol. Chem*. **266**, 16818-16824.

Williams, M. A. and Fukuda, M. (1990). Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J. Cell Biol*. **111**, 955-966.

Wood, S. A. and Brown, W. J. (1992). The morphology, but not the function of endosomes and lysosomes is altered by brefeldin-A. *J. Cell Biol*. **119**, 273-285.

Zerial, M. and McBride, H. (2001). Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* **2**, 107-117.