Autotaxin/Lysophospholipase D-mediated Lysophosphatidic Acid Signaling Is Required to Form Distinctive Large Lysosomes in the Visceral Endoderm Cells of the Mouse Yolk Sac*

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Autotaxin, a lysophospholipase D encoded by the Enpp2 gene, is an exoenzyme that produces lysophosphatidic acid in the extracellular space. Lysophosphatidic acid acts on specific G-protein-coupled receptors, thereby regulating cell growth, migration, and survival. Previous studies have revealed that Enpp2−/− mouse embryos die at about embryonic day (E) 9.5 because of angiogenic defects in the yolk sac. However, what cellular defects are caused by the lack of LPA production is not clear.

Lysosphospholipase D (LPLD) is a major LPA-producing enzyme (9, 10). LPA is a lipid mediator possessing a wide variety of biological functions, including cell proliferation, migration, and survival (4–6). LPA activates LPA1–6 receptors, which are coupled to several downstream signals via at least three distinct G protein subfamilies as follows: G12/13, Gi, and Gq (4, 5, 7). Additionally, the role of autotaxin in sphingosine 1-phosphate (S1P) production was suggested in vitro but remains to be established in vivo (4, 8). Recently, it has been reported that Enpp2−/− mice died at embryonic day (E) 9.5 because of angiogenic defects in the yolk sac (9, 10). Enpp2−/− embryos also showed allantois malformation, neural tube defects, no axial turning, and head enlargement, indicating the essential roles of Enpp2 for mouse embryonic development (9, 10). Furthermore, adult Enpp2-heterozygous mice showed half-normal levels of lysophospholipase D activity and LPA but normal levels of S1P in plasma, suggesting that autotaxin is a major LPA-producing enzyme in vivo (9, 10). However, what cellular defects are caused by the lack of LPA production in Enpp2−/− embryos and what signaling pathways underlie the defects remain elusive.

The extraembryonic visceral yolk sac is the first place where hematopoiesis and vasculogenesis occur in the mouse. It is composed of two layers, the visceral endoderm (VE) and the underlying mesoderm layers. VE cells play a critical role in the materno-fetal exchange of nutrients prior to the establishment of a chorioallantoic placenta (~E9). They endocytose maternal proteins vigorously, hydrolyze the proteins in lysosomes, and supply the resultant products to the developing embryo (11, 12). Reflecting high endocytic and digestive activity, VE cells are endowed with distinctively large lysosomes (known as amorphous vacuoles in electron microscopy) (11, 12). Blockade of VE cell functions using chemicals, antibodies, or gene disruption caused fetal malformation (11, 13), suggesting the functional importance of VE cells in rodent development.

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cytic, and autophagic pathways (14, 15). They undergo dynamic fusion/fission, and yet cells maintain a relatively constant size and number of lysosomes (14, 15). Genetic studies in yeast and Drosophila as well as cell biological studies in mammalian cells have identified many intracellular components involved in the biogenesis of lysosomes and vacuoles (yeast homologs of lysosomes) and elucidated their functions (14, 15). However, it remains unknown whether lysosome biogenesis is regulated by extracellular signals and which signaling pathway(s), if any, regulate the process.

In this study, we show that Enpp2 mRNA is highly expressed in the yolk sac VE cells from E7.5 to E9.5. In the VE cells of Enpp2−/− embryos, distinctive large lysosomes are fragmented. When embryos are cultured in the presence of pharmacological inhibitors for the LPA receptors, Rho, ROCK/Rho kinase, or LIM kinase (LIMK), these drugs induce lysosomal defects similar to those observed in Enpp2−/− VE cells. In addition, inhibition of Rho, ROCK, or LIMK by electroporating their dominant negative forms or activation of cofilin by electroporating its constitutively active form into wild-type embryos induces the size reduction of lysosomes in VE cells. In Enpp2−/− VE cells, the steady-state levels of cofilin phosphorylation and actin polymerization are reduced. Moreover, perturbations of actin turnover dynamics by cytochalasin B or jasplakinolide result in the defect in lysosome formation in VE cells. These findings suggest that the control of actin turnover dynamics through the Rho–ROCK–LIMK pathway by way of autotaxin-LPA signaling is required for the regulatory processes of lysosome biogenesis.

EXPERIMENTAL PROCEDURES

Animal Experiments—All the experiments using animals were approved by the Animal Care and Use Committee of the University of Tsukuba and performed under its guidelines. Noon of the day on which a vaginal plug was observed was taken as embryonic day 0.5. Timed pregnant ICR mice (CLEA Japan, Tokyo, and Japan SLC, Hamamatsu, Japan) were used for pharmacological experiments and electroporation.

Generation of Enpp2-deficient Mice—A gene-targeting vector was constructed by inserting the mouse genomic DNA fragments flanking exons 6 and 7 of the Enpp2 gene into a TC3 vector (a gift from Dr. Ryoichiro Kageyama, Kyoto University, Tokyo, and Japan SLC, Hamamatsu, Japan) which contained a cassette of stop-IRES-lacZ-poly(A), a neomycin-resistant gene, and a diphtheria toxin A fragment (a gift from Dr. Ryoichiro Kageyama, Kyoto University, Japan), which contained a cassette of stop-IRES-lacZ-poly(A), a neomycin-resistant gene, and a diphtheria toxin A fragment (a gift from Dr. Ryoichiro Kageyama, Kyoto University, Japan), which contained a cassette of stop-IRES-lacZ-poly(A), a neomycin-resistant gene, and a diphtheria toxin A fragment (a gift from Dr. Ryoichiro Kageyama, Kyoto University, Japan), which contained a cassette of stop-IRES-lacZ-poly(A), a neomycin-resistant gene, and a diphtheria toxin A fragment (a gift from Dr. Ryoichiro Kageyama, Kyoto University, Japan), which contained a cassette of stop-IRES-lacZ-poly(A), a neomycin-resistant gene, and a diphtheria toxin A fragment (a gift from Dr. Ryoichiro Kageyama, Kyoto University, Japan), which contained a cassette of stop-IRES-lacZ-poly(A), a neomycin-resistant gene, and a diphtheria toxin A fragment (a gift from Dr. Ryoichiro Kageyama, Kyoto University, Japan), which contained a cassette of stop-IRES-lacZ-poly(A), a neomycin-resistant gene, and a diphtheria toxin A fragment (a gift from Dr. Ryoichiro Kageyama, Kyoto University, Japan), which contained a cassette of stop-IRES-lacZ-poly(A), a neomycin-resistant gene, and a diphtheria toxin A fragment (a gift from Dr. Ryoichiro Kageyama, Kyoto Un...
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RESULTS

Formation of Large Lysosomes Is Impaired in Enpp2−/− VE Cells—Enpp2 knock-out mice were generated by disrupting the exons that encode the enzyme catalytic domain (Fig. 1, A and B). Enpp2 mRNA and protein were completely absent in Enpp2−/− mice (Fig. 1, C and D). Although heterozygous mice were apparently normal and fertile, Enpp2−/− embryos died at E9.5 because of angiogenic defects in the yolk sac (Fig. 1, E and F), as reported previously (9, 10). To elucidate the roles of Enpp2 in early development, its expression before E9.5 was examined. Enpp2 mRNA was first detectable in the embryonic ectoderm at the late cylinder and primitive streak stages (E6.0 to E6.5) (Fig. 2A). From E7.5 to E8.5, strong signals were detected in the yolk sac, especially in VE cells (Fig. 2A). RT-PCR indicated that Enpp2 mRNA was highly expressed in the yolk sac between E7.5 and E9.5 (Fig. 2B).

Thus, we focused on yolk sac VE cells. While searching for possible defects using immunohistochemistry, we noticed intriguing changes in the intracellular vesicles in Enpp2−/− VE cells. Because VE cells vigorously take up maternal proteins, including IgG from the apical surface, endocytic vesicles can be visualized simply by immunostaining for mouse IgG. Control VE cells had large IgG-containing vesicles, whereas Enpp2−/− VE cells had smaller punctate vesicles (Fig. 3A, top). Electron microscopy demonstrated that control VE cells had a few large vesicles in the apical portion, whereas Enpp2−/− VE cells had numerous smaller vesicles (Fig. 3A, middle). To examine lysosomes in VE cells, we stained whole embryos with LysoTracker Red, a fluorescent dye that selectively accumulates in acidic organelles, and observed lysosomes in VE cells using a confocal microscope. This analysis revealed that lysosomes became significantly smaller, and the number of lysosomes significantly increased in Enpp2−/− VE cells (Fig. 3, A and B). Similarly, immunostaining with a lysosome marker, LAMP1, also revealed that lysosomes were smaller in Enpp2−/− VE cells (supplemental Fig. S1). These findings indicate that Enpp2−/− embryos had defects in the formation of large lysosomes, which are one of the distinctive features of VE cells.

LPA Receptors Are Required to Form Large Lysosomes in VE Cells—We next examined the downstream signaling pathways of autotaxin using an ex vivo whole embryo culture system. Whole embryo culture is a well established system that has been proved to be useful for studying normal development as well as assessing teratogenicity and gene functions (17, 18). When E7.5 wild-type embryos were cultured for 1 day, the appearances of the yolk sac and embryos were indistinguishable from those of the in utero-grown E8.5 wild-type embryos (supplemental Fig. S2A). In addition, when E7.5 Enpp2−/− embryos were cultured for 1 day, head cavity was formed as seen in the in utero-grown E8.5 Enpp2−/− embryos (supplemental Fig. S2A). Furthermore, the lysosome size in VE cells was reduced in the cultured Enpp2−/− embryos compared with that in the cultured wild-
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![Diagram of lysosome biogenesis](image)

**FIGURE 1. Targeted disruption of the murine Enpp2 gene.** A, scheme of targeting strategy. Part of exon 6 and the entirety of exon 7 of the Enpp2 gene were replaced with a stop-IRES-lacZ-poly(A)-pGK-neo cassette. The abbreviations used are as follows: DTA, diphtheria toxin A fragment; H, HpaI; P, PvuII, S, SacI, B, Southern blotting of HpaI-, PvuII-, or SacI-digested genomic DNA hybridized with 5', 3', or neo probes verified the germ line transmission of the correctly targeted Enpp2 allele. Closed and open arrowheads indicate the bands for wild-type and targeted alleles, respectively. The band sizes of the wild-type and targeted alleles are 8.6 and 6.8 kb for the 5’ probe and 7.0 and 4.5 kb for the 3’ probe, respectively. C, RT-PCR analysis revealed that Enpp2 mRNA was absent in the Enpp2−/− embryos. WT indicates Enpp2+/+, or Enpp2+/− control embryos (denoted in the same way hereafter). D, Western blot revealed that autotaxin protein was absent in the extraembryonic coelomic fluid of the E8.5 Enpp2−/− embryos. WT indicates Enpp2+/+ or Enpp2+/− control embryos (denoted in the same way hereafter). E, appearance of the E9.5 yolk sac. Large vitelline vessels formed in the control (black arrow) but not in the Enpp2−/− yolk sac, in which red blood cells precipitated (white arrows). F, gross appearance of the E9.5 embryos. Most Enpp2−/− embryos were much smaller than the controls and did not undergo embryonic turning. The arrow indicates the head cavity in Enpp2−/− embryos. Scale bar represents 1 mm.

Type embryos, in the same way and to the same extent that the lysosome size in VE cells was reduced in the Enpp2−/− embryos in vivo (supplemental Fig. S2, A and B). These results indicate that a whole embryo culture system faithfully mimics in vivo development of wild-type and Enpp2−/− embryos, thus providing a feasible tool to test the effects of pharmacological manipulation of VE cells.

We thus set out to test the effects of the inhibitors of the LPA signaling pathway. In this study, we used subtoxic concentrations of drugs, which were carefully predetermined in preliminary tests as not inducing developmental retardation or cardiac arrest (data not shown). First, LPA and S1P receptor antagonists were tested. When E7.5 wild-type embryos were cultured for 1 day with 10 μM Ki16425, an LPA1/LPA3 antagonist (24), lysosomes became significantly smaller (Fig. 4, A and B). In contrast, 10 μM VPC23019, an S1P1/S1P3 antagonist (25), did not induce size reduction of lysosomes (Fig. 4, A and B), although the drug must have effectively acted on the yolk sac because it induced angiogenic defects (data not shown). These findings indicate that LPA receptor signaling is required to form large lysosomes in VE cells.

**Rho-ROCK Pathway Is Required to Form Large Lysosomes in VE Cells**—LPA receptors are coupled to several signaling pathways through distinct G proteins; the major pathways include G12/13-Rho-ROCK, Gq-phosphatidylinositol 3-kinase-Rac, Gq-Ras-MAPK, and Gq-phospholipase C (4, 5, 7). To determine which pathway is required downstream of autotaxin, we tested selective inhibitors for each pathway. Treatment of embryos with either a Rho inhibitor, Clostridium C3 exoenzyme (20 μg/ml), or a ROCK inhibitor, H1152 (0.1 μM), resulted in a significant size reduction of lysosomes (Fig. 4C and supplemental Fig. S3A). Other ROCK inhibitors, hydroxyfasudil (1–10 μM) and Y-27632 (1 μM), were also effective (supplemental Fig. S4). In contrast, none of the inhibitors of Gq (pertussis toxin, 0.5 μg/ml), phospholipase C (U-73122, 10 μM), phosphatidylinositol 3-kinase (LY294002, 10 μM), or MAPK (PD98059, 10 μM) had any effect on the size of lysosomes (Fig. 4C and supplemental Fig. S3A). These findings indicate that the Rho-ROCK pathway plays an important role downstream of autotaxin-LPA signaling in the formation of large lysosomes in VE cells.

**LIMK Pathway Is Required to Form Large Lysosomes in VE Cells**—The main downstream targets of ROCK are LIMK and myosin light chain. ROCK phosphorylates and activates LIMK, which in turn phosphorylates and inactivates cofilin, a protein that stimulates actin filament disassembly (23, 26, 27). ROCK also induces myosin light chain phosphorylation directly and indirectly through the inhibition of myosin phosphatase by phosphorylating its myosin-binding subunit (28). We tested which pathway is the main target of ROCK in this system. When mouse embryos were cultured with a myosin II inhibitor, blebbistatin (10 μM), the lysosome size was not changed (Fig. 4D and supplemental Fig. S3B). Thus, we next examined the effect of a cell-permeable LIMK inhibitor, S3 peptide, which contains the N-terminal 16-amino acid sequence of cofilin and the cell-permeable sequence of penetratin (29). As a negative
control, the reverse (RV) peptide, which contains the reverse sequence of cofilin and the cell-permeable sequence of penetra- titin, was used (29). When mouse embryos were cultured with 15 μg/ml S3 or RV peptide, S3 peptide inhibited cofilin phosphor- ylation (supplemental Fig. S5) and resulted in the size reduction of lysosomes in VE cells (Fig. 4 and supplemental Fig. S3). These findings indicate that the LIMK-cofilin pathway is the main target of ROCK and responsible for the formation of large lysosomes in VE cells.

If LIMK activity is reduced in Enpp2−/− VE cells, it would be expected to decrease phosphorylation of cofilin (23, 26, 27). We thus examined phosphorylation levels of cofilin by immuno- staining of VE cells with anti-phosphorylated cofilin or anti- total cofilin antibody. Quantitation of the staining intensity revealed that the steady-state level of cofilin phosphorylation was significantly decreased without affecting the cofilin levels in Enpp2−/− VE cells (Fig. 5, A and B). This finding of decreased cofilin phosphorylation strongly supports the supposition that LIMK activity was decreased in Enpp2−/− VE cells.

Actin Filaments Are Depolymerized in Enpp2−/− VE Cells
— Reduced phosphorylation of cofilin activates cofilin, thereby leading to depolymerization and severance of the actin fila-
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A

B

C

D

E

Figure 4. Blockade of the LPA receptor-Rho-ROCK-LIMK pathway induces defects in lysosome formation. A, confocal microscopic images of the yolk sac stained with LysoTracker Red. After E7.5 wild-type embryos were cultured with an LPA$_1$/LPA$_3$ antagonist (10 nM Ki16425) or an S1P$_1$/S1P$_3$ antagonist (10 nM VPC23019) for 1 day, LysoTracker Red staining was performed. Ki16425 induced the size reduction of lysosomes in the VE cells, whereas VPC23019 was ineffective. Scale bar represents 6 μm. B–E, quantitation of the lysosome size in the embryos cultured with the indicated inhibitors. Treatment of wild-type embryos with Ki16425 (Ki) as well as the inhibitors of Rho (20 μg/ml C3 exoenzyme), ROCK (0.1 μM H1152), or LIMK (15 μg/ml S3 peptide) resulted in significant size reduction in lysosomes when compared with the controls, whereas treatment with VPC23019 (VPC) and the inhibitors for G$_i$ (0.5 μg/ml pertussis toxin (PTX)), phospholipase C (10 μg/ml U-73122), phosphatidylinositol 3-kinase (10 μg/ml LY294002), MAPK (10 μg/ml PD98059), or myosin II (10 μg/ml blebbistatin) showed no effect on the size of lysosomes. The values are the means ± S.E. *, p < 0.05; **, p < 0.01; analysis of variance with a Tukey-Kramer post hoc test (B and C) and unpaired t test (D and E). The number of embryos examined is shown in each column. RV, the reverse peptide used as a negative control.

To examine whether this happens in Enpp2$^{−/−}$ VE cells, we stained whole embryos with Alexa488-phalloidin and visualized actin filaments in VE cells using a confocal microscope. As predicted from the above findings, the amounts of polymerized actin (observed as the phalloidin staining intensity in confocal microscopy) appeared to be reduced throughout Enpp2$^{−/−}$ VE cells compared with those in the wild-type VE cells (Fig. 6A). To exclude the possibility that the differences in the staining intensity resulted from the difference in the Alexa488-phalloidin permeability, we performed the staining using the sections of the yolk sac. In this experiment, to compare the signals precisely, we obtained the fluorescence images using a confocal microscope with constant scanning parameters. As shown in Fig. 6B, phalloidin staining intensity was lower in the Enpp2$^{−/−}$ VE cells than that in the wild-type cells. Statistical analysis of the fluorescence intensity in the yolk sac sections indicated that the amounts of polymerized actin were significantly lower in Enpp2$^{−/−}$ VE cells than those in wild-type VE cells (Fig. 6C).

Next, we examined whether actin polymerization in VE cells was regulated by the LPA receptor-Rho-ROCK-LIMK pathway. We cultured wild-type mouse embryos with the inhibitors used in the above experiments for 1 day and performed phalloidin staining. As a result, all the drugs that induced the reduction of the lysosome size (Ki16425, C3 exoenzyme, H1152, and S3 peptide) resulted in reductions in phalloidin staining in VE cells (Fig. 6D). Thus, the amount of polymerized actin was reduced in Enpp2$^{−/−}$ VE cells most likely as a result of the decrease in the LPA receptor-Rho-ROCK-LIMK signaling.

The above findings suggest the possibility that actin filaments regulate lysosome formation in VE cells. To test this, we examined directly whether perturbation of actin cytoskeleton dynamics causes defects in lysosomes. First, to induce actin-depolymerized conditions similar to those in Enpp2$^{−/−}$ VE cells, E7.5 embryos were cultured with 0.3 μM cytochalasin B for 1 day. This treatment led to a significant reduction in lysosome size in VE cells (Fig. 7A), suggesting that actin polymerization is required to form large lysosomes. Moreover, when the embryos cultured with cytochalasin B for 1 day were observed after the drug was removed, the lysosome sizes in VE cells returned to normal over time (Fig. 7B). This finding indicates that the effects of cytochalasin B on lysosomes were reversible and not merely cytotoxic actions. Reversely, stabilization of actin filaments by culturing embryos with 10 nM jasplakinolide also induced reduction in lysosome size (Fig. 7A). Thus, actin depolymerization is also required for lysosome formation. Taken together, these findings suggest that the dynamic regulation of actin turnover is required for the formation of large lysosomes in VE cells, whereas actin filaments tend to shift to the net depolymerization because of the decrease of cofilin as a result of steady-state dephosphorylation of cofilin in Enpp2$^{−/−}$ VE cells.

Electroporation of Dominant Negative Forms of Rho, ROCK, or LIMK Induces Lysosomal Defects in VE Cells—Finally, we tested the validity of our pharmacological experiments using a molecular approach; we overexpressed DN or CA forms of Rho, ROCK, LIMK, or cofilin in VE cells, and we observed the changes in lysosome size. For this purpose, we developed a method to introduce exogenous DNAs into VE cells by electroporation (see details under “Experimental Procedures”). The DN forms used were RhoB (T19N), ROCK (KD-1A), LIMK1 (D460A), and cofilin (S3E), whereas the CA forms used were RhoB (G14V), ROCK (D3), LIMK1 (T508EE), and cofilin (S3A) (19–23). To label the electroporated cells, Rho and LIMK cDNAs were subcloned into a pEGFP-IRES vector, whereas ROCK and cofilin were fused to EGFP and yellow fluorescent protein, respectively.
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We first examined whether inhibition of the Rho-ROCK-LIMK pathway by electroporating their DN forms induced depolymerization of actin filaments. After E7.5 whole embryos were dissected and electroporated, they were cultured for 1 day using an ex vivo whole embryo culture system and subsequently stained with Alexa546-phalloidin. When EGFP alone was electroporated, phalloidin staining intensity was not changed (Fig. 8A), indicating that neither electroporation nor EGFP overexpression affected actin polymerization. In contrast, electroporation of DN-Rho, DN-ROCK, or DN-LIMK induced actin depolymerization (Fig. 8A), indicating that the Rho-ROCK-LIMK pathway was inhibited by the electroporation of these expression constructs.

Next, we examined the effects of Rho-ROCK-LIMK inhibition on lysosome formation using LysoTracker Red staining. Electroporation of EGFP alone showed no obvious effects on lysosome size, whereas electroporation of DN-Rho, DN-ROCK, or DN-LIMK reduced the size of lysosomes in VE cells (Fig. 8B–C). In severe cases (10–20%), LysoTracker Red staining disappeared (data not shown) (Fig. 8C). Inhibition of Rho-ROCK-LIMK pathway results in the decrease of coflin phosphorylation, thus leading to activation of coflin. To mimic this, we used the S3A mutant of coflin, which is constitutively active and cannot be phosphorylated. Electroporation of coflin (S3A) induced the size reduction of lysosomes, although the steady-state levels of phalloidin-staining intensity were not apparently changed (Fig. 8). Thus, these findings demonstrate that inhibition of the Rho-ROCK-LIMK-cofilin signaling pathway led to defects in the formation of large lysosomes in VE cells.

Next, we examined the effects of Rho-ROCK-LIMK activation on lysosome formation. When the CA forms of Rho or LIMK were electroporated, actin polymerization was increased (supplemental Fig. S6A), indicating that Rho and LIMK were activated in VE cells by electroporation of these CA constructs. CA-Rho and CA-LIMK led to the reduction of lysosome size in VE cells (supplemental Fig. S6, B and C), suggesting that activation of Rho or LIMK resulted in the defects in lysosome formation. When CA-Rock was electroporated, VE cells became severely deformed and were unsuitable for further examination (data not shown). We thus excluded CA-Rock electroporation from the analysis. Activation of the Rho-ROCK-LIMK pathway phosphorylates coflin, thereby leading to inactivation of coflin. To induce this state, we used the S3E mutant of coflin, which mimics the phosphorylated state of coflin. When coflin (S3E) was electroporated, lysosomes became smaller, although the changes in the steady-state levels of phalloidin staining were not apparent (supplemental Fig. S6, B and C). These findings suggest that overactivation of the Rho-ROCK-LIMK-cofilin pathway led to the defects in lysosome formation. Thus, the balance between actin polymerization and depolymerization was important to maintain the large size of lysosomes in VE cells, compatible with our findings that both cytochalasin B and jasplakinolide led to lysosomal defects.

DISCUSSION

In this study, we demonstrated that autotaxin-LPA signaling is required to form distinctive large lysosomes in yolk sac VE cells. We provided the evidence that constitutive activation of LPA receptors as a result of high Enpp2 expression in the yolk sac VE cells, starting from E7.5, is required for the formation of large lysosomes. We also showed that actin turnover dynamics regulated by the Rho-ROCK-LIMK pathway play a central role in the downstream pathway.

We found linkage between autotaxin-LPA signaling and lysosome biogenesis. Although little is known about the relationship between LPA and organelle biogenesis, multiple roles of Rho GTPases, the main downstream effectors of LPA signaling, in endocytic trafficking have been reported (30, 31). For example, RhoB and RhoD, GTPases known to associate with endosomes, control endocytic trafficking by regulating actin polymerization (30, 31). The interaction of RhoB and mDia2 on endosomes regulates vesicle trafficking by controlling actin dynamics (32). Our findings demonstrate that inhibition of Rho and its downstream signaling pathway by pharmacological blockers or electroporation of DN constructs led to fragmentation of lysosomes in VE cells. Thus, our results suggest that extracellular production of LPA by autotaxin in mouse embryos controls endocytic vesicle trafficking through the Rho pathway to form large lysosomes in yolk sac VE cells. This notion is compatible with a
previous argument that the phenotypes of Enpp2−/− embryos are similar to those of embryos lacking Gα12 and Gα13, the key mediators for the Rho pathway (10, 33).

We also showed linkage between autotaxin and actin dynamics. Although it is well known that LPA regulates actin cytoskeleton rearrangements, ours is the first study to indicate that autotaxin controls the levels of actin polymerization in vivo. Because autotaxin protein was detected in the ECF (Fig. 1D), autotaxin should produce LPA and activate LPA receptors constantly in mouse embryos, thereby controlling actin dynamics required for large lysosome formation in VE cells. In addition to the well studied roles of actin in endocytosis (34–36), accumulating evidence has suggested that actin directly regulates vesicle fusion and transport in the endocytic pathway (34, 35). For example, treatment of cells with actin-depolymerizing drugs induced defects in the transport from late endosomes to lysosomes in mammalian cells (37, 38). In addition, in vitro fusion assays showed that actin facilitated fusion between phagosomes and late endosomes and between late endosomes themselves (39). Thus, autotaxin appears to promote vesicle fusion and/or transport processes from endosomes to lysosomes in VE cells. Future studies are required to examine whether and how autotaxin-LPA controls endocytic vesicle trafficking. It may be also necessary to understand the roles of autotaxin-LPA from another perspective of LPA as a lipid. It is well known that localized changes in the lipid composition regulate the membrane curvature and are required for vesicle formation and membrane fission (40, 41). For example,
LPA and phosphatidic acid are interconverted enzymatically, and the chemical properties of LPA and phosphatidic acid favor opposite membrane curvatures (40, 41). Therefore, LPA production by autotaxin may play a role in lysosome biogenesis by controlling membrane dynamics in addition to the regulation of actin remodelling through LPA receptor signaling.

VE cells and yeast have many common features in endocytic vesicles. First, both are active in endocytosis/digestion and have large lysosomes (vacuoles). Second, Rho activity is required for lysosome (vacuole) formation; two Rho GTPases, Rho1p and Cdc42p, are enriched on vacuole membranes and required for docking and fusion of vacuoles in yeast (42), whereas in VE cells, C3 exoenzyme and DN-Rho induced fragmentation of lysosomes. Third, actin is involved in lysosome formation; actin was bound to vacuoles, and vacuole fusion was inhibited by jasplakinolide or an actin destabilizing reagent, latrunculin, in yeast (43), whereas cytochalasin B or jasplakinolide inhibited lysosome formation in VE cells. Therefore, there seems to be a common cellular machinery to maintain large lysosomes in these cells. We tried to enlarge lysosomes in cultured cell lines by treating them with LPA, by overexpressing Enpp2, or by transfecting CA forms of Rho, ROCK, or LIMK, but we could not see any changes in lysosome size.3 Thus, to make such large lysosomes (vacuoles) as observed in VE cells and yeast, sufficient amounts of certain cellular components required for lysosome biogenesis may be necessary in addition to the activation of Rho signaling and actin dynamics. Conversely, we tried to reduce the size of lysosomes in cultured cell lines by treating them with LPA antagonists or by transfecting DN forms of Rho, ROCK, or LIMK. However, we did not see any apparent changes of lysosome size in VE cells, although we could not evaluate the lysosome size correctly because lysosomes in cultured cell lines were much smaller than those in VE cells.

In summary, we have found a novel function of autotaxin-LPA signaling in the regulation of lysosome biogenesis. Further studies are required to examine whether autotaxin-LPA controls actin dynamics and lysosome formation in other Enpp2-expressing cells, exactly which processes in lysosome biogenesis are regulated by autotaxin-LPA, and what biological significance can be attributed to the autotaxin-LPA signaling in lysosome formation. It is also worth noting that yol

3 S. Koike and M. Masu, unpublished observations.
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sac VE cells provide a useful system to study the cellular and molecular mechanisms of vesicle trafficking and biogenesis in mammalian cells because of the simple anatomy of the yolk sac, the large size and easy labeling of endocytic vesicles, and their accessibility for pharmacological manipulation and a molecular approach.

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