Lysosomes are organelles that play a crucial role in the degradation of endocytosed molecules, phagocytosed macromolecules and autophagic substrates. The membrane of lysosomes contains several highly glycosylated membrane proteins, and lysosome-associated membrane protein (LAMP)-1 and LAMP-2 account for a major portion of the lysosomal membrane glycoproteins. Although it is well known that LAMP-2 deficiency causes Danon disease, which is characterized by cardiomyopathy, myopathy and mental retardation, the roles of lysosomal membrane proteins including LAMP-1 and LAMP-2 in myogenesis are not fully understood.

In this study, to understand the role of LAMP proteins in the course of differentiation of myoblasts into myotubes, we used C2C12 myoblasts and found that the protein and mRNA levels of LAMP-1 and LAMP-2 were increased in the course of differentiation of C2C12 myoblasts into myotubes. Then, we investigated the effects of LAMP-1 or LAMP-2 knockdown on C2C12 myotube formation, and found that LAMP-1 or LAMP-2 depletion impaired the differentiation of C2C12 myoblasts and reduced the diameter of C2C12 myotubes. LAMP-2 knockdown more severely impaired C2C12 myotube formation compared with LAMP-1 knockdown, and knockdown of LAMP-1 did not exacerbate the suppressive effects of LAMP-2 knockdown on C2C12 myotube formation. In addition, knockdown of LAMP-1 or LAMP-2 decreased the expression levels of myogenic regulatory factors, MyoD and myogenin. These results demonstrate that both LAMP-1 and LAMP-2 are involved in C2C12 myotube formation and LAMP-2 may contribute dominantly to it.

**Key words**  C2C12 myoblast; differentiation; lysosomal glycoprotein

Lysosomes are acidic organelles that play a crucial role in the degradation of endocytosed molecules, phagocytosed macromolecules and autophagic substrates. Lysosomes contain soluble acid-dependent hydrolases and possess highly glycosylated integral membrane proteins, and lysosome-associated membrane protein (LAMP)-1 and LAMP-2 are known as major lysosomal membrane glycoproteins. Both LAMP-1 and LAMP-2 consist of an highly glycosylated luminal domain, a single transmembrane domain and a short C-terminal cytoplasmic tail, and the cytoplasmic tail possesses a tyrosine-based sorting signal, which is recognized by adaptor proteins and participates in their transport to lysosomes.

Mutations in genes encoding lysosomal membrane proteins cause human diseases, and it is known that LAMP-2 deficiency causes Danon disease, which is characterized by cardiomyopathy, myopathy and mental retardation. Previous reports have shown that LAMP-2 knockout mice exhibit extensive accumulation of autophagic vacuoles in many tissues, including liver, pancreas, spleen, kidney, skeletal muscle and heart, whereas LAMP-1 knockout mice exhibit a relatively mild phenotype. It has also been reported that LAMP proteins are involved in lysosome-phagosome fusion.

Skeletal muscle plays roles in locomotion and energy metabolism, and myogenesis, the process of generating muscle, is required for the development of skeletal muscle. Myogenesis includes various cellular events, such as determination, migration, proliferation and differentiation, and during the differentiation process, myoblasts exit cell cycle and fuse to form multinucleated myotubes. Accumulating evidence indicates that the four myogenic regulatory factors (MRFs), MyoD, Myf5, myogenin and MRF4, are involved in the control of myogenesis. MyoD and Myf5 are required for the determination of skeletal myoblasts, while myogenin is implicated in the regulation of differentiation process and MRF4 has both determination and differentiation functions. The MRFs possess a conserved basic helix-loop-helix domain that mediates dimerization with E proteins and recognizing E-box consensus sequence, and binding of MRFs to E-box consensus sequence activates the expression of genes involved in myogenesis.

Previous reports have demonstrated that the expression and activity of lysosomal cathepsins increase during differentiation of myoblasts into myotubes, indicating a role of lysosomal proteins in the process of differentiation of myoblasts into myotubes. It was also reported that LAMP-2 knockout mice exhibit autophagic vacuole accumulation in skeletal muscles. However, the roles of lysosomal membrane proteins including LAMP-1 and LAMP-2 in the course of differentiation of myoblasts into myotubes are not fully investigated. In this study, to understand the role of LAMP proteins in the course of differentiation of myoblasts into myotubes, we carried out LAMP-knockdown experiments in C2C12 myoblasts. We found that the protein and mRNA levels of LAMP-1 and LAMP-2 were increased during differentiation of C2C12 myoblasts into myotubes, and that both LAMP-1 and LAMP-2 participated in differentiation of C2C12 myoblasts. We also found that LAMP-1 knockdown did not exacerbate the suppressive effects of LAMP-2 knockdown on C2C12 myotube formation. Our results provide information available for understanding the role of LAMP-1 and LAMP-2 in the process of differentiation of myoblasts into myotubes.
MATERIALS AND METHODS

Antibodies An anti-myosin heavy chain (MHC) antibody (MAB4470, 1:500 and 1:5000 for immunocytochemistry and Western blotting, respectively) was purchased from R&D Systems (Minneapolis, MN, U.S.A.). Anti-α-tubulin (PM054, 1:2500 for Western blotting) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; M171-3, 1:2500 for Western blotting) antibodies were purchased from MBL (Nagoya, Japan). Anti-LAMP-1 (1D4B) and anti-LAMP-2 (ABL93) antibodies were kindly provided by Dr. Thomas August (Johns Hopkins University). Anti-MyoD (sc-377460, 1:300 for Western blotting) and anti-myogenin (sc-12732, 1:300 for Western blotting) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). An anti-LGP85 antibody was prepared as described previously.

Cell Culture and Transfection C2C12 myoblast cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with l-glutamine (Wako), penicillin–streptomycin (Wako) and 20% fetal bovine serum. To differentiate the myoblasts into myotubes, the culture medium was replaced with DMEM supplemented with l-glutamine, penicillin–streptomycin and 2% horse serum (hereafter referred to as differentiation medium (DM)). To knockdown LAMP-1 or LAMP-2, C2C12 cells were transfected with 20nm small interfering RNA (siRNA) using RNAiMAX (Thermo Fisher Scientific, Waltham, MA, U.S.A.) according to the manufacturer’s protocol. Negative control (12935-300), LAMP-1 #1 (CCCACAUCAGCAUCUCCACCACU), LAMP-1 #2 (GGUAAUUAUCAGACUCUCAAUCU), LAMP-1 #4 (UGGCGUCUAGGACUCUGACUGA), LAMP-2 #1 (GACAGAUUCAAGGGAUUCGUCC), LAMP-2 #2 (UCUCGGGUUAAAAGGGCGCAAGCUC), and LAMP-2 #3 (CCUCAUCUCCUGUUCUGCCGAAUAU) stealth siRNAs were purchased from Thermo Fisher Scientific.

Western Blotting To observe the expression of proteins, C2C12 cells were lysed in the buffer containing 1% NP-40, 137mM NaCl and 20mM Tris–HCl (pH 8.0), and the protein concentration of cell lysates was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific). Then, the cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, U.S.A.) and probed with primary antibodies. Then, the membrane was incubated with hors eradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.). Clarity Western ECL substrate (Bio-Rad, Hercules, CA, U.S.A.) was used to detect immunoreactive bands with a LuminoGraph II (ATTO, Tokyo, Japan). Quantification of band intensity was performed using CS Analyzer 4 (ATTO).

Immunocytochemistry C2C12 cells grown on glass coverslips were fixed in phosphate-buffered saline (PBS) containing 3.7% formaldehyde, and then the cells were permeabilized with PBS containing 0.2% (w/v) Triton X-100 and 0.2% bovine serum albumin. The cells were treated with an anti-MHC antibody for 1h and washed three times with PBS, and then the cells were treated with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (Thermo Fisher Scientific) for 1h. Nuclei of the cell was stained with 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Kumamoto, Japan). Cells were observed using a Leica TCS-SPE DMi4000B (Leica Microsystems, Wetzlar, Germany). The differentiation index was defined as the percentage of the number of nuclei in MHC-positive cells to the total number of nuclei, and three fields were chosen randomly to measure the differentiation index. To determine the diameter of C2C12 myotubes, six fields were chosen randomly, and 10 myotubes per field were selected for measurement. Ten measurements along each myotube were performed using LAS-AF software, and the diameter was defined as the mean of these 10 measurements.

RT-PCR NucleoSpin RNA II (TaKaRa, Shiga, Japan) was used to isolate total RNA from C2C12 cells. The isolated RNA sample (1µg) was reverse transcribed using a TaKaRa PrimeScript RT reagent kit (TaKaRa) according to the manufacturer’s protocol. Quantitative RT-PCR was performed using a Light Cycler 2.0 (Roche Diagnostics). The reverse transcription products were amplified in a reaction mixture (20µL) containing SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa) and 0.5µM of each primer. The forward and reverse primers were as follows: mouse LAMP-1, GTCTCTATGCCTCCTATG; mouse LAMP-2, AAGGTGCAACTTATGTGAC; mouse myogenin, GAGCACCACAAAGTACTCAG; mouse GAPDH, GAGCACCACAAAGTACTCAG. The mRNA levels were measured using Student’s t-test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

The Expression of LAMP Proteins Increases in the Course of Differentiation of C2C12 Myoblasts into Myotubes To explore whether LAMP-1 and LAMP-2 participate in the course of differentiation of myoblasts into myotubes, we utilized mouse C2C12 myoblasts that are generally used to study the molecular mechanism of myogenesis. C2C12 myoblasts fuse to form multinucleated C2C12 myotubes when cultured in DM. In our investigation into the function of LAMP-1 and LAMP-2 using C2C12 cells, we found that the protein levels of LAMP-1 and LAMP-2 increased in the course of differentiation of C2C12 myoblasts into myotubes (Fig. 1A). The protein level of MHC, a marker of differentiation, we performed RT-PCR analysis. The mRNA level of LAMP-1 increased during C2C12 differentiation (Fig. 1B). The mRNA level of LAMP-2 was also increased on days 1 and 2, but was not significantly elevated on days 3 and 4, when compared with that on day 0 (Fig. 1B). To evaluate whether the protein and mRNA expression levels of other lysosomal membrane proteins also increase during C2C12 differentiation, we investigated the expression of LGP85/LIMP II, another well-known lysosomal membrane protein.
protein and mRNA expression levels of LGP85 increased during C2C12 differentiation (Supplementary Fig. S1). These results indicate that the protein and mRNA levels of LAMP-1, LAMP-2 and LGP85 increase during differentiation of C2C12 myoblasts into myotubes.

Knockdown of LAMP-1 or LAMP-2 Impairs C2C12 Myoblast Differentiation Because the protein and mRNA levels of LAMPS increased in the process of C2C12 differentiation...
ation, it is possible that both LAMP-1 and LAMP-2 participate in C2C12 myotube formation. To understand whether LAMP-1 is involved in the differentiation of C2C12 myoblasts, we investigated the differentiation index (the percentage of the number of nuclei in MHC-positive cells to the total number of nuclei) in C2C12 cells incubated with control or LAMP-1-targeting siRNAs and cultured in DM for 3 d. The LAMP-1 protein level was remarkably decreased in C2C12 cells treated with LAMP-1-targeting siRNAs (Fig. 2A). The differentiation index in control cells was 34.0%, whereas it was lower in C2C12 cells treated with LAMP-1 #1, LAMP-1 #2 or LAMP-1 #4 siRNAs (26.8, 28.8 and 30.2%, respectively; Figs. 2B, C). These data suggest that LAMP-1 participates in the differentiation of C2C12 myoblasts.

We also investigated the effect of LAMP-2 knockdown on C2C12 myoblast differentiation. The LAMP-2 protein level was significantly lowered in C2C12 cells treated with LAMP-2-targeting siRNAs (Fig. 3A). After C2C12 myoblasts incubated with control or LAMP-2-targeting siRNA were cultured in DM for 3 d, the differentiation index was investigated. The differentiation index in control cells was 34.0%, whereas it was lower in C2C12 cells treated with LAMP-2 #1, LAMP-2 #2 or LAMP-2 #3 siRNAs (26.8, 28.8 and 30.2%, respectively; Figs. 3B, C). These data suggest that LAMP-2 is also related to the differentiation of C2C12 myoblasts.

The Effect of Simultaneous Knockdown of LAMP-1 and LAMP-2 on C2C12 Myoblast Differentiation

Because involvement of either LAMP-1 or LAMP-2 in C2C12 myoblast differentiation is implied by the results of the above experiments, it is of interest to know if combined knockdown of LAMP-1 and LAMP-2 has a synergistic or an additive effect. Therefore, we simultaneously depleted LAMP-1 and LAMP-2 in C2C12 myoblasts, and investigated the differentiation index. When C2C12 myoblasts were transfected with LAMP-1- and LAMP-2-targeting siRNAs and cultured in DM for 3 d, the protein levels of LAMP-1 and LAMP-2 were remarkably decreased (Fig. 4A). After C2C12 myoblasts were transfected with LAMP-1- and LAMP-2-targeting siRNAs and cultured in DM for 3 d, the differentiation index was determined. The differentiation index in control cells was 34.5%, whereas it was lower in C2C12 cells transfected with LAMP-1 #1, LAMP-2 #3, or both LAMP-1 #1 and LAMP-2 #3 siRNAs (29.0, 23.0 and 23.1%, respectively; Figs. 4B, C). These results suggest that LAMP-2 knockdown more severely impaired the differentiation of C2C12 myoblasts compared with LAMP-1 knockdown. Neither a synergistic nor an additive effect of LAMP-1 and LAMP-2 knockdown on C2C12 myoblast differentiation was observed.

The Effect of LAMP-1 and LAMP-2 Knockdown on C2C12 Myotube Diameter

We further investigated whether LAMP-1 or LAMP-2 knockdown decreases the diameter of C2C12 myotubes. The diameter of the C2C12 myotubes in
Fig. 4. The Effect of Simultaneous Knockdown of LAMP Proteins on C2C12 Myoblast Differentiation

(A) C2C12 myoblasts transfected with control, LAMP-1 #1, LAMP-2 #3, or both LAMP-1 #1 and LAMP-2 #3 siRNAs were cultured in DM for 3 d, and the expression levels of indicated proteins were investigated. (B) C2C12 myoblasts transfected with control, LAMP-1 #1, LAMP-2 #3, or both LAMP-1 #1 and LAMP-2 #3 siRNAs were cultured in DM for 3 d. Then the cells were immunostained with an anti-MHC antibody followed by DAPI staining (MHC, green; DAPI, blue). Representative areas of three independent experiments are shown. Scale bars, 100 µm. (C) The differentiation indexes of C2C12 cells in (B) were calculated, and are presented as the means±S.E.M. from three independent experiments. Con, control. *p<0.05; ns, not significant by a Student’s t-test. (Color figure can be accessed in the online version.)

The diameters of C2C12 myotubes transfected with control siRNA were taken as 1.0. Transfection with LAMP-1 #1, LAMP-2 #3, or both LAMP-1 #1 and LAMP-2 #3 siRNAs decreased the diameter of myotube to 0.82, 0.70 and 0.69, respectively (Fig. 5). These data suggest that LAMP-2 knockdown more severely decreased the diameter of C2C12 myotubes compared with LAMP-1 knockdown, and that LAMP-1 knockdown did not exacerbate the suppressive effect of LAMP-2 knockdown on the diameter of C2C12 myotubes.

The Effect of LAMP Knockdown on the Expression of MyoD and Myogenin

To obtain clues for understanding a mechanism by which LAMP knockdown affects C2C12 differentiation, we investigated whether LAMP knockdown causes altered expressions of MRFs, such as MyoD and myogenin in C2C12 cells. C2C12 myoblasts treated with control or LAMP siRNAs were cultured in DM for 3 d, and then the mRNA level of MyoD and myogenin was measured. When the mRNA level of MyoD and myogenin in control cells was taken as 1.0, knockdown of LAMP-1, LAMP-2, or both LAMP-1 and LAMP-2 decreased the mRNA level of MyoD to 0.83, 0.71 and 0.73, respectively (Fig. 6A). Furthermore, knockdown of LAMP-1, LAMP-2, or both LAMP-1 and LAMP-2 decreased the mRNA level of myogenin to 0.85, 0.77 and 0.79, respectively (Fig. 6A). LAMP-2 knockdown more severely...
decreased MyoD and myogenin mRNA levels compared with LAMP-1 knockdown, while additive or synergistic effects of LAMP-1 knockdown and LAMP-2 knockdown on MyoD and myogenin mRNA levels were not observed (Fig. 6A). We next investigated the MyoD and myogenin protein levels in C2C12 cells cultured in DM for 3 d after the siRNA treatment. When the protein level of MyoD and myogenin in control cells was taken as 1.0, depletion of LAMP-1, LAMP-2, or both LAMP-1 and LAMP-2 decreased the protein level of MyoD to 0.95, 0.84 and 0.81, respectively (Figs. 6B, C). Furthermore, knockdown of LAMP-1, LAMP-2, or both LAMP-1 and LAMP-2 decreased the protein level of myogenin to 0.85, 0.85 and 0.84, respectively (Figs. 6B, C). Although LAMP-2 knockdown more lowered the protein level of MyoD than LAMP-1 knockdown, LAMP-1, LAMP-2 and both knockdown caused almost the same decrease in the myogenin protein level (Figs. 6B, C). These results show that LAMP knockdown affects the expression of MyoD and myogenin in C2C12 cells.

**DISCUSSION**

Lysosomes play a crucial role in the degradation of many endocytosed molecules, phagocytosed macromolecules and autophagic substrates. Because mutations in genes encoding lysosomal membrane proteins induce a variety of human diseases, it is thought that lysosomes are not simply the terminal of the degradation pathway, but are implicated in diverse cellular functions. 2,3) Our results demonstrate that both LAMP-1 and LAMP-2 contribute to C2C12 myotube formation. Although either LAMP-1 or LAMP-2 knockdown impaired C2C12 myoblast differentiation and decreased the diameter of C2C12 myotubes, LAMP-1-knockdown cells exhibited a milder phenotype than LAMP-2-knockdown cells. Furthermore, LAMP-1 depletion did not enhance the suppressive effect of LAMP-2 depletion on C2C12 myotube formation. These data propose that LAMP-2, rather than LAMP-1, plays a pivotal role in C2C12 myotube formation.
It was previously demonstrated that LysoTracker fluorescence, GFP-LC3B puncta and LC3B-II protein increased during C2C12 differentiation. These results imply that the lysosomal content and autophagosome formation increase during C2C12 differentiation. Furthermore, it was reported that the expression and activity of lysosomal cathepsins increased during C2C12 differentiation, indicating a role of lysosomal proteins in myoblast differentiation. Consistent with these results, the protein and mRNA levels of LAMP-1, LAMP-2 and LGP85 were all increased in the process of C2C12 differentiation. The increase in the three major lysosomal membrane proteins is likely linked to an augmentation in the lysosomal content during conversion of C2C12 myoblasts into myotubes. A previous study has shown that transcription factor EB (TFEB), a member of microphthalmia-transcription factor E (MiT/TFE) subfamily of basic helix-loop-helix leucine zipper transcription factor, binds to Coordinated Lysosomal Expression and Regulation (CLEAR) element and is involved in the expression of various genes encoding lysosomal proteins. The promoter region of LAMP-1 gene possesses multiple CLEAR elements and LAMP-1 increases by the TFEB overexpression, suggesting that TFEB is involved in the expression of LAMP-1 during lysosomal biogenesis. Therefore, it is plausible that TFEB is related to the increased expression of LAMP-1 during C2C12 differentiation. The expression of LAMP-2 and LGP85 increased during C2C12 differentiation as well, but the expression of genes encoding these two proteins was not up-regulated by the TFEB overexpression, implying that molecular mechanisms underlying the augmented expression of LAMP-1 and LAMP-2 during C2C12 differentiation may differ.

Considering our immunocytochemical results and the effect of LAMP knockdown on the expression of MRFs together, it is suggested that LAMP-1 and LAMP-2 knockdown suppress the induction of MyoD and myogenin, resulting in the impairment of C2C12 myoblast differentiation and a reduction in the diameter of C2C12 myotubes. The mRNA and protein levels of MyoD and the mRNA level of myogenin were more severely decreased in LAMP-2 knockdown cells cultured in DM for 3 d compared with LAMP-1 knockdown cells cultured in DM for 3 d. Meanwhile, knockdown of LAMP-1 or LAMP-2 decreased the protein level of myogenin to similar degree, implying that LAMP-1 knockdown may affect the protein stability of myogenin and decrease it to the similar level to LAMP-2 knockdown at the later stage of C2C12 differentiation. Inhibition of lysosomal function induces translocation of TFEB and its two closely related transcription factors, TFE3 and microphthalmia-associated transcription factor (MiTF) to the nucleus. Among these proteins, TFE3 overexpression and microphthalmia-associated transcription factor (MITF) to TFE3 and its two closely related transcription factors, TFE3 and microphthalmia-associated transcription factor (MiTF) to the nucleus. Among these proteins, TFE3 overexpression and microphthalmia-associated transcription factor (MITF) to TFE3 overexpression, 34,35) indicating a role of lysosomal proteins in myoblast differentiation. Furthermore, it was reported that LAMP-1 and LAMP-2 knockdown both induced cytoplasmic accumulation of the autophagc vacuole marker LC3-GFP in HeLa cells; that the autophagy/lysosome system seems to be involved in the control of muscle mass; and that the pathogenic mechanism of Danon disease seems to be related to the impaired autophagosome–lysosome fusion. Therefore, it is possible that autophagic vacuoles also accumulated in LAMP-1- and LAMP-2-depleted C2C12 cells, and that impaired autophagosomes–lysosome fusion was involved in the suppressive effects of LAMP-1 and LAMP-2 knockdown on C2C12 myotube formation.

Alternative splicing of LAMP-2 produces three isoforms: LAMP-2A, LAMP-2B and LAMP-2C. Among the three LAMP-2 isoforms, LAMP-2B is abundantly expressed in skeletal muscle, and LAMP-2B deficiency causes Danon disease. Because the LAMP-2 siRNAs used in this study were designed to target all three isoforms, it is not clear which LAMP-2 isoform is required for proper differentiation at present. Therefore, it remains to be elucidated whether LAMP-2B is a crucial isoform involved in C2C12 myoblast differentiation in vitro.

Acknowledgments We thank Drs. Jun Kamishikiryo, Hiroshi Matsuoka and Masatoshi Ohnishi for valuable comments. This work was supported by the Research Center for Green Science, Fukuoyama University to H.S.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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