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Phospholipase C-related catalytically inactive protein, a novel microtubule-associated protein 1 light chain 3-binding protein, negatively regulates autophagosome formation

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ABSTRACT

Upon starvation, cells undergo autophagy, an intracellular bulk-degradation process, to provide the required nutrients. Here, we observed that phospholipase C-related catalytically inactive protein (PRIP) binds to microtubule-associated protein 1 light chain 3 (LC3), a mammalian autophagy-related initiator that regulates the autophagy pathway. Then, we examined the involvement of PRIP in the nutrient depletion-induced autophagy pathway. Enhanced colocalization of PRIP with LC3 was clearly seen in nutrient-starved mouse embryonic fibroblasts under a fluorescent microscope, and interaction of the proteins was revealed by immunoprecipitation experiments with an anti-LC3 antibody. Under starvation conditions, there were more green fluorescent protein fused-LC3 dots in mouse embryonic fibroblasts from *PRIP*-deficient mice than in fibroblasts from wild type cells. The formation of new dots in a single cell increased, as assessed by time-lapse microscopy. Furthermore, the increase in autophagosome formation in *PRIP*-deficient cells was notably inhibited by exogenously overexpressed PRIP. Taken together, PRIP is a novel LC3-binding protein that acts as a negative modulator of autophagosome formation.

1. Introduction

Macroautophagy (often referred to as autophagy) is an intracellular degradation pathway in eukaryotic cells in which cytoplasmic components are delivered to a degradative organelle, the lysosome, wherein the components are degraded. Stress conditions, such as nutrient starvation, induce autophagy, and autophagy provides cells with nutrients by degrading cytoplasmic materials [1]. Moreover, autophagy maintains intracellular homeostasis by degrading harmful aggregated proteins and damaged organelles and protects humans from many diseases, including cancer, neurodegenerative and cardiovascular disorders, and infectious diseases [1,2].

Upon induction of autophagy, a double- or multiple-layered membrane appears, extends around the target substrates, and ultimately fuses on both ends to become a vesicle (autophagosome). Microtubule-associated protein 1 light chain 3 (LC3) is essential for this process. LC3 is proteolytically cleaved (LC3-I) and lipidated to achieve its active form (LC3-II). To date, LC3-II is the only known protein that specifically associates with autophagosomes [3,4]. There are several mammalian homologs of Atg8, a ubiquitin-like protein required for yeast autophagosome formation. These homologs include LC3A, LC3B, LC3C, $GABA_A$ receptor-associated protein (GABARAP), glandular epithelial cell protein 1 (GEC1; GABARAP-like 1), the 16-kDa Golgi-associated ATPase enhancer (GATE-16; GABARAP-like 2), and GABARAP-like 3. However, the stage of autophagy at which each mammalian Atg8 homolog participates is still unclear [5].

Phospholipase C-related catalytically inactive protein type 1 (PRIP1) was first identified as a novel D-*myo*-inositol 1,4,5-trisphosphate-binding protein, similar to phospholipase C-δ1 but catalytically inactive and expressed predominantly in the brain [6–10]. Later, a second isoform, PRIP2, with a relatively broad

Abbreviations: a.a., amino acid residues; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution; GABARAP, $GABA_A$ receptor-associated protein; GFP, green fluorescent protein; *GFP-LC3* WT, GFP-LC3-expressing WT; GST, glutathione *S*-transferase; His, histidine$_6$; LC3, microtubule-associated protein 1 light chain 3; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin; PRIP, phospholipase C-related catalytically inactive protein; *PRIP*-KO, *PRIP1* and *PRIP2* double knockout; WT, wild type.

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distribution in tissues, including the brain, was reported [11–13]. We identified GABARAP, which modulates $GABA_A$ receptor intracellular trafficking [14], as a PRIP-binding partner [15]. The finding helped elucidate the roles of PRIP in $GABA_A$ receptor signaling [15–17]. We have also identified additional PRIP-binding proteins: $GABA_A$ receptor β subunits [18–19], catalytic subunit of protein phosphatase 1 [20–23], protein phosphatase 2A [18,23], and phospho-Akt/protein kinase B [24]. Based on these findings, we concluded that PRIP mediates the phosphodependent modulation of $GABA_A$ receptor function and trafficking and modulates SNAP-25-phosphoregulated exocytosis by regulating protein phosphatase 1, protein phosphatase 2A, and phosphorylated Akt [18,21,24,25].

In this study, because PRIP interacted with the GABARAP mammalian homologue LC3, we investigated the involvement of PRIP in LC3-mediated autophagy. We observed enhanced autophagosome formation in *PRIP1* and *PRIP2* double-knockout (*PRIP*-KO) cells. We propose PRIP as a novel LC3-binding protein that inhibits autophagosome formation.

2. Materials and methods

2.1. Antibodies

Previously described anti-PRIP1 rabbit polyclonal antibodies were used [15]. Anti-LC3 rabbit polyclonal antibody for western blotting (#2775) was purchased from Cell Signaling Technology (Danvers, MA). Anti-LC3 mouse monoclonal antibody (M152-3 clone 4E12) for the immunoprecipitation assay, anti-p62 rabbit polyclonal antibody (PM045), and rabbit control IgG were all purchased from MBL (Nagoya, Japan). Anti-actin mouse monoclonal antibody (A5316 clone AC-74) from Sigma–Aldrich (St. Louis, MO), and horseradish peroxidase-conjugated secondary antibodies from GE Healthcare Life Sciences (Uppsala, Sweden), were used.

2.2. Animals

PRIP-KO mouse strains and the corresponding wild-type (WT) mice were generated as described previously [16]. The green fluorescent protein (GFP)-*LC3* transgenic mouse strain (GFP-LC3#53) was obtained from RIKEN BioResource Center (Tsukuba, Japan) [4]. These mice were mated to obtain *PRIP*-KO mice stably expressing GFP-LC3 and WT mice stably expressing GFP-LC3 (*GFP-LC3* WT). The Animal Care Committee of Kyushu University and Hiroshima University, following the guidelines of the Japanese Council on Animal Care, approved the handling of animals as well as all the procedures.

2.3. Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) were prepared from WT and *PRIP*-KO mice and from WT and *PRIP*-KO mice expressing GFP-LC3 at embryonic day 13.5 by a standard method. MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin G, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator.

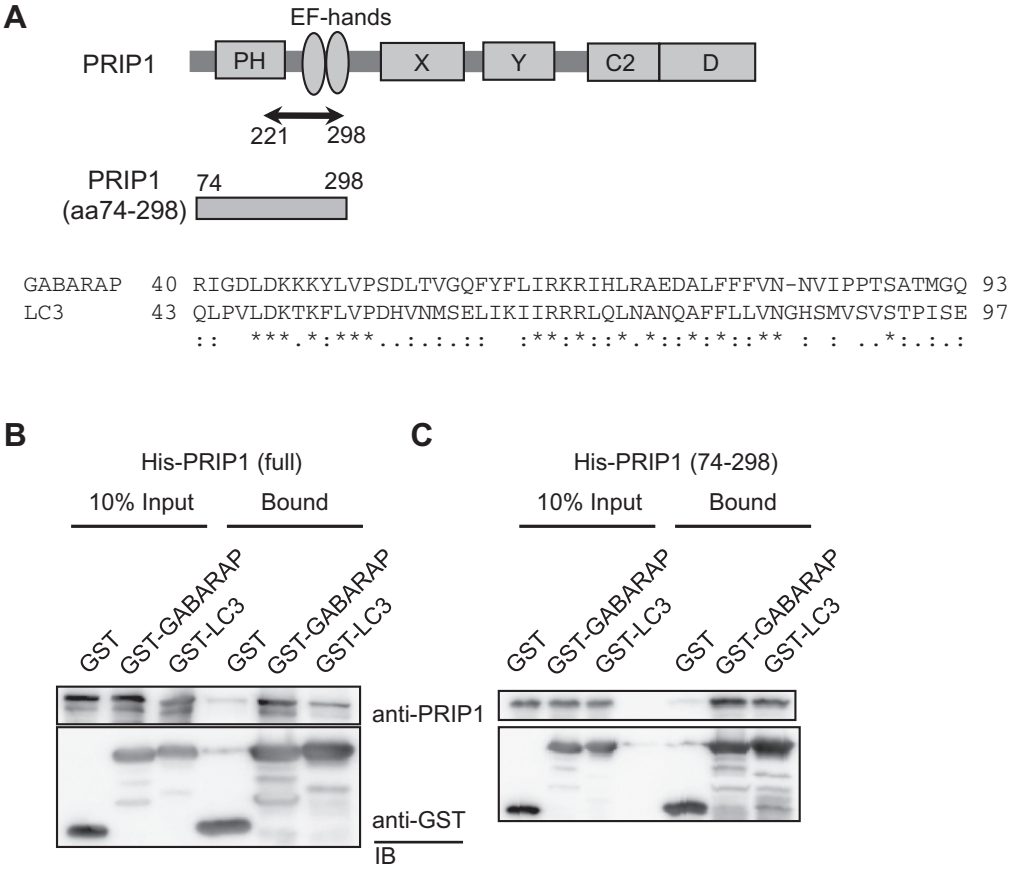

Fig. 1. Association between PRIP and LC3. (A) Schematic representation of rat PRIP1 and the truncated mutant (top). The amino acid sequences of the PRIP-binding regions in GABARAP and LC3 are shown (bottom). The numbers indicate the amino acid residues. The arrow represents the GABARAP-binding site on PRIP1. Conserved (*), highly similar (:), and similar (.) residues are indicated. PH, pleckstrin homology domain; EF hand, EF hand-like motif; X and Y, distorted triose phosphatase isomerase barrel-like domains; C2, C2 domain; D, assumed rigid domain structure. (B and C) GST pull-down assays. His-tagged PRIP1 (full-length) (B) or His-tagged PRIP1 (74–298) (C) was incubated with GST, GST-GABARAP, and GST-LC3, and pull down assays were performed. Precipitates were analyzed by immunoblotting (IB) by using anti-PRIP1 and anti-GST antibodies. The blots shown are representative of images from at least three independent experiments that yielded similar results.

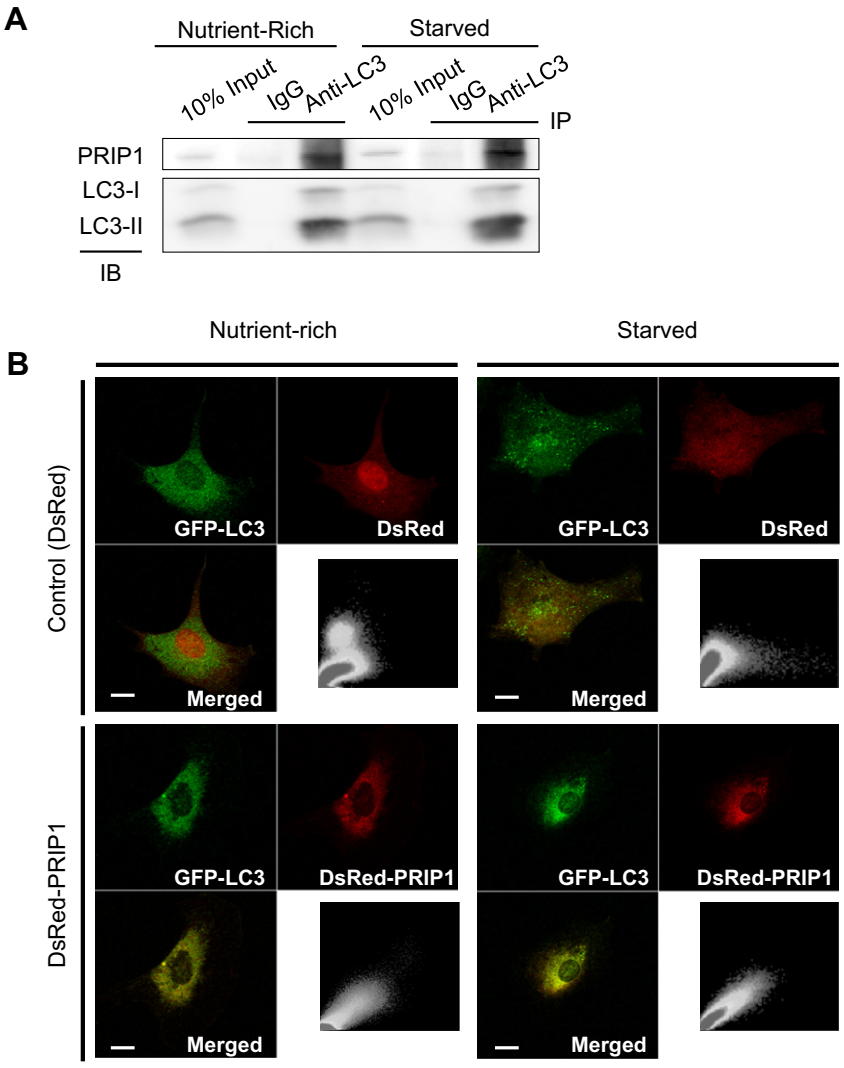

Fig. 2. Interaction between PRIP1 and LC3 in MEFs. (A) WT MEFs were cultured under nutrient-rich or starvation conditions for 2 h. Cell lysates were collected and immunoprecipitated (IP) by anti-LC3 and control IgG antibodies. Immunoblotting (IB) was performed with the anti-PRIP1 and anti-LC3 antibodies. We obtained similar images from three independent experiments, and a representative image is shown. (B) *GFP-LC3* WT MEFs were transiently transfected with vector (DsRed) or DsRed-PRIP1. On the following day, the cells were cultured under nutrient-rich (left) or starvation (right) conditions for 2 h and then fixed. A typical image of each experiment taken with a Zeiss LSM510 META confocal microscope is shown; green, red, and yellow represent the GFP-LC3, DsRed vector or DsRed-PRIP1, and merged images, respectively (scale bar: 10 μm). Correlation plots (horizontal, green intensity; vertical, red intensity) derived from each colocalized image, analyzed by the Zeiss LSM 510 META software ver4.0, are also shown (lower right in each panel). Four or five randomly chosen fields were analyzed in three independent experiments. At least 15 cells for each experiment were observed.

DsRed-PRIP1 plasmid [18] transfection was performed using a Gene Pulser electroporation system (Bio-Rad, Hercules, CA), according to the manufacturer's protocol.

2.4. Immunoprecipitation and glutathione S-transferase (GST) protein pull-down assays

MEFs were lysed in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% TritonX-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, and a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 100 μM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 3.4 μg/mL aprotinin). The lysates were immunoprecipitated using the indicated antibodies [24]. The GST pull-down assay was performed as described previously [15]. Briefly, GST-LC3, GST-GABARAP, and GST immobilized on glutathione-Sepharose™ 4B (GE Healthcare) were incubated with histidine$_6$ (His)-tagged PRIP1 and His-tagged PRIP1 (74–298) [26,27] at 4 °C for 2 h. Precipitates were separated by SDS–polyacrylamide gel electrophoresis and analyzed by western blotting with anti-PRIP1 antibody.

2.5. Western blotting

Samples obtained from immunoprecipitation and GST-pull down experiments were analyzed by western blot using the indicated antibodies. Immunoreactive signals were detected using the Immobilon™ western detection system (Millipore, Billerica, MA) and the LAS-3000 mini imaging system (Fuji Film, Tokyo, Japan).

2.6. Autophagosome formation assay

MEFs stably expressing GFP-LC3 were seeded on glass coverslips in DMEM containing 10% fetal bovine serum (nutrient-rich). The following day, the medium was replaced with fresh nutrient-rich culture medium at 4 h before the experiments. Then, autophagy

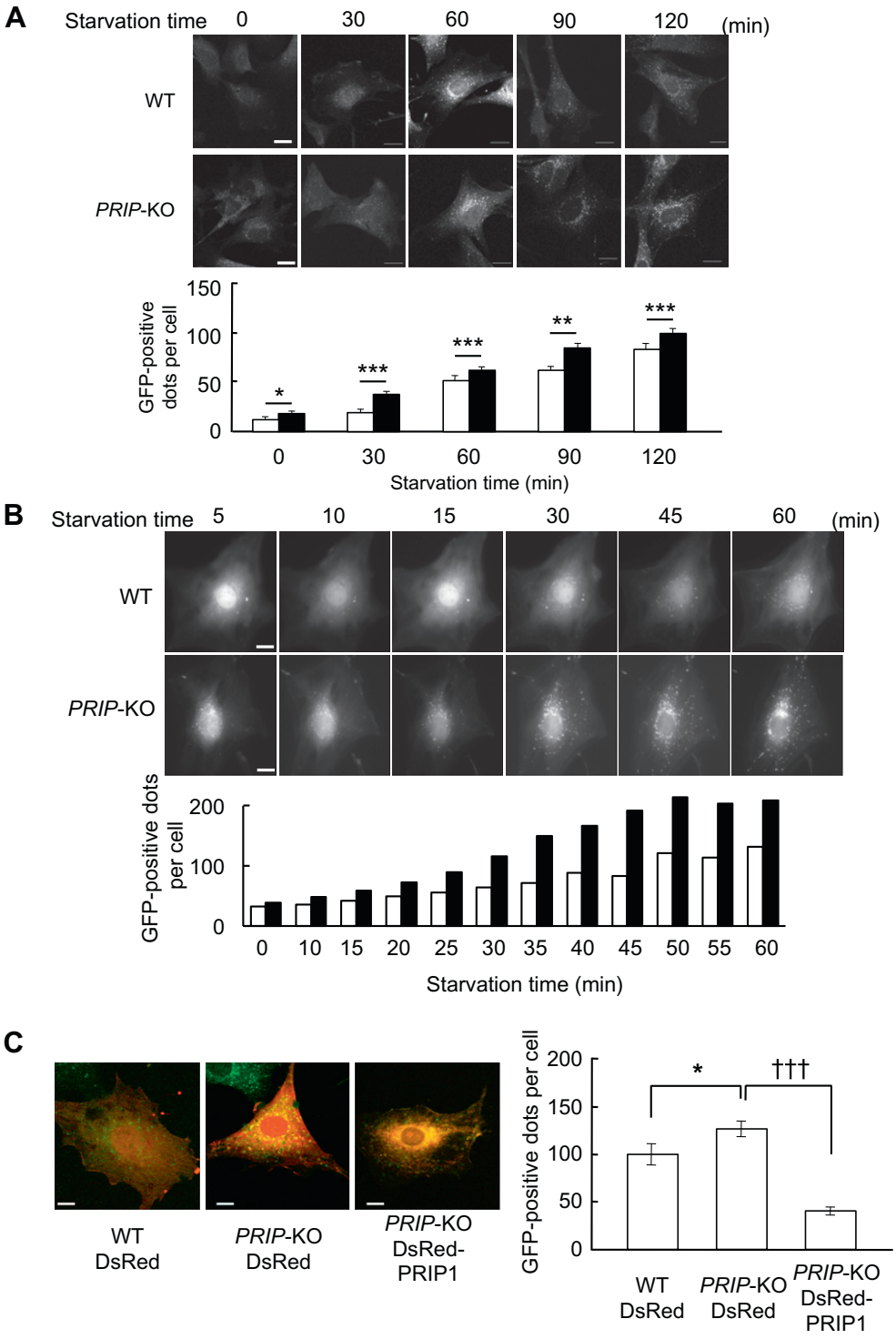

Fig. 3. Autophagic activities in WT and *PRIP*-KO MEFs. (A) The GFP-LC3 dot-formation assay was performed to analyze the number of autophagosomes per cell. *GFP-LC3* WT and *PRIP*-KO MEFs were incubated with 10% FBS/DMEM (nutrient-rich) or EBSS (starvation) for the indicated times and fixed. Images were obtained from similar small cells by confocal fluorescence microscopy. The graph (white and black bars: WT and *PRIP*-KO, respectively) shows the quantification of the number of GFP-LC3 puncta. Data are presented as mean ± SE values (Student's *t*-test, WT versus *PRIP*-KO: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). At least 10 fields in five independent experiments gave similar images. (B) Time-lapse analyses of the dynamics of autophagosome formation in MEFs expressing GFP-LC3 during nutrient starvation. A set of fluorescent images, acquired using confocal microscopy over 60 min at the indicated intervals, is shown. We randomly chose similar large cells from both genotypes. We repeated this experiment three times and obtained similar results. A typical image is shown. The graph shows the quantification of the number of GFP-LC3 puncta obtained from the represented images. (C) Overexpression of exogenous PRIP1 in *PRIP*-KO MEFs. *PRIP*-KO MEFs were transfected with DsRed (mock) or DsRed-PRIP1, and the number of GFP puncta (autophagosomes) was quantified (right panel). The confocal microscope images show GFP-LC3 puncta under the starvation condition. Data are presented as the mean ± SE values (ANOVA, WT versus *PRIP*-KO: $^{*}p < 0.05$; *PRIP*-KO versus DsRed-PRIP1: $^{\dagger\dagger\dagger}p < 0.001$). Five fields in three independent experiments gave similar images (scale bar in A, B, and C: 10 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was induced by Earle's balanced salt solution (EBSS; E3024, Sigma) for the indicated times. Cells were fixed with 4% paraformaldehyde for 5 min and rinsed well with phosphate-buffered saline. Cells were mounted and visualized by confocal microscopy (Zeiss LSM510 META). To quantify GFP-LC3-positive autophagosomes, 3–5 different confocal microscopy images were randomly chosen, and GFP-positive dots were examined on the images with identical brightness and contrast settings. GFP-positive dots with a diameter of >500 nm were counted as GFP-LC3 autophagosomes. To analyze colocalization, merged images of GFP-LC3 and DsRed or DsRed-PRIP1 signals were analyzed (Zeiss LSM510 META ver4.0). Time-lapse images were obtained using a fluorescence microscope IX81 (Olympus) with a cooled charge-coupled device camera (Roper Scientific), and the images were analyzed using the NIH ImageJ software (National Institute of Health). GFP-dots were counted independently by two observers.

3. Results

3.1. Association of PRIP with LC3

PRIP binds to GABARAP at the amino acid residues (a.a.) 40–93 [15], which is highly conserved in LC3 (a.a. 43–97) (see Fig. 1A). Then, we first examined whether PRIP also interacts with LC3 *in vitro*. GST pull-down assays were performed using combinations of GST-fused GABARAP and LC3, and His-tagged PRIP1. As shown in Fig. 1B, GST-GABARAP and GST-LC3, but not GST alone, bound full-length PRIP1. The binding with LC3 appeared slightly less than that with GABARAP. We previously mapped the 221–298 of PRIP1 as GABARAP-binding region (Fig. 1A) [15]. We also tested the association with LC3 or GABARAP by using a short version of PRIP1 (Fig. 1C). Short PRIP1 (a.a. 74–298) was found to be associated with GST-GABARAP and GST-LC3. The binding of the short PRIP1 to LC3 appeared slightly greater than that of full-length PRIP1, indicating that full-length PRIP1 provides some steric hindrance for the association.

3.2. Colocalization of PRIP with GFP-LC3

Since LC3 is known to be an autophagy-related protein, we posited a role for PRIP in autophagy regulation. To examine the association between PRIP and LC3 in living cells, we performed an immunoprecipitation assay with anti-LC3 antibody by using cell lysates from WT-MEFs in both nutrient-rich and starvation conditions. Subsequent immunoblotting with an anti-PRIP1 antibody showed specific binding of PRIP1 to LC3 in living cells under both conditions (Fig. 2A).

We generated control WT and *PRIP*-KO mice that expressed GFP-LC3 and prepared MEF cell lines. We first examined the colocalization of PRIP1 with LC3 under nutrient-rich and starvation conditions. WT *GFP-LC3* MEFs were transiently transfected with a DsRed-PRIP1 plasmid or the empty vector and observed by confocal microscopy. As shown in the upper panels of Fig. 2B, in DsRed vector-transfected cells, GFP-LC3 dot formation was observed in response to nutrient starvation (upper right), but there was little correlation between the green dot fluorescence and the red vector signals that appeared diffusely in cytosol (see the correlation plot). On the other hand, under starvation conditions, less GFP-LC3 dot formation was observed in GFP-LC3 cells with DsRed-PRIP1 overexpression (lower panels) than in vector-transfected control cells (compare the upper and lower right panels). Furthermore, we observed colocalization between green (LC3) and red (PRIP1) signals under nutrient-rich conditions, and the colocalization was enhanced in response to starvation (see the correlation plots). These results indicate that DsRed-PRIP1 colocalized with GFP-LC3 under nutrient-rich conditions in MEFs, and that the association was enhanced by starvation. Thus, the association between PRIP1 and LC3 may negatively regulate starvation-induced LC3 dot formation.

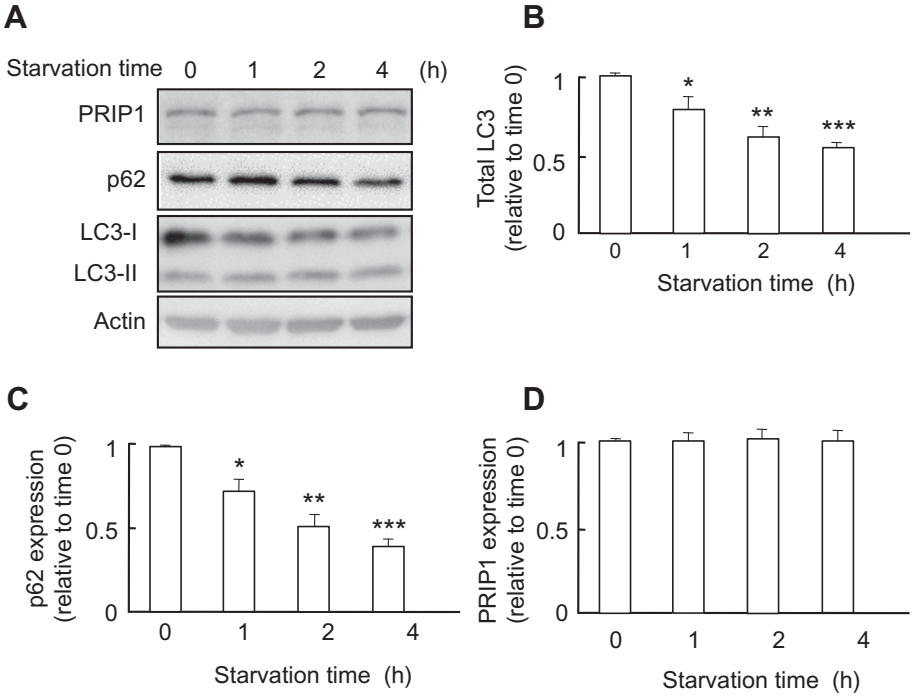

Fig. 4. PRIP is not an autophagy substrate. WT MEFs were incubated with the starvation medium for the indicated times. A representative western blot image with each of the indicated antibodies is shown (A). Degradation assays show the expression levels of LC3 (B), p62 (C), and PRIP1 (D), relative to actin at the indicated times. Data are represented as the mean ± SE values (ANOVA, amount at each time versus amount at time 0 (set to 1): $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, $n = 3$).

3.3. Elevated autophagosome formation in PRIP-KO MEFs

To investigate the effect of PRIP on autophagy, autophagosome-formation assays were performed. *GFP-LC3* WT and *GFP-LC3 PRIP*-KO MEFs were examined for the formation of GFP-positive dots after the starvation (Fig. 3A). Under the nutrient-rich condition (time 0), the number of dots in *PRIP*-KO cells was slightly higher than the number of dots in WT MEFs ($p < 0.05$). As the starvation time lengthened (~2 h), the number of GFP-positive dots gradually increased in both WT and *PRIP*-KO cells, but the number in *PRIP*-KO MEFs remained slightly higher (Fig. 3A graph). The results suggest that autophagic activity was elevated in *PRIP*-KO cells, indicating an inhibitory function(s) of PRIP in the autophagic machinery.

We next monitored the process of dot formation in live cells by using time-lapse microscopy and quantified the appearance of new dots every 5 min during nutrient depletion (Fig. 3B). The number of GFP-positive dots increased over time in MEFs from both genotypes. New GFP-positive dots appeared at a higher frequency in *PRIP*-KO MEFs than in WT MEFs (Fig. 3B graph). These results indicate that autophagosome formation in *PRIP*-deficient cells increased.

To test the involvement of PRIP in autophagosome formation, we overexpressed DsRed-PRIP1 in *PRIP*-KO *GFP-LC3* MEFs and assessed GFP-positive dot formation after nutrient starvation for 2 h. As shown in Fig. 3C, PRIP deficiency increased the number of dots by approximately 30%, while exogenous expression of PRIP1 decreased the number of dots by approximately 70%, suggesting that PRIP negatively regulates autophagy.

3.4. PRIP1 is not an autophagy substrate

Since PRIP interacted with LC3 and colocalized to the autophagosomes, we examined whether PRIP1 could be degraded by autophagy by using HEK cells expressing PRIP1 (Fig. 4A). The amounts of LC3 and the LC3-binding protein p62, a selective autophagy substrate also known as SQSTM1/sequestome 1 [28,29], decreased during 4 h of starvation (Fig. 4B, C). However, the amount of PRIP1 did not decrease (Fig. 4D), suggesting that PRIP is not an autophagy substrate.

4. Discussion

Although we have learned much about the molecular mechanism underlying autophagy, some intriguing questions are remaining. One is what regulates autophagosome formation in response to nutrient starvation. Here, we investigated a new protein involved in autophagosome formation.

In this study, we identified PRIP as a novel LC3-binding protein based on the highly conserved amino acid homology between LC3 (a.a. 43–97) and GABARAP (a.a. 40–93). PRIP has two isoforms in mammals, PRIP1 and PRIP2. The ability of PRIP2 to associate with LC3 was confirmed by a GST pull-down assay (data not shown). PRIP1 and PRIP2 are not known to have functional differences, although their organ distribution differs: PRIP1 exhibits limited expression chiefly in the brain, whereas PRIP2 exhibits a ubiquitous expression pattern (for review, see [30]). When PRIP1 and PRIP2 were detected in MEFs by western blotting, PRIP1 expression predominated (data not shown). Therefore, we studied the effect of PRIP1 on the autophagy pathway by using MEFs prepared from WT and *PRIP*-KO mice.

Starvation slightly enhanced the coimmunoprecipitation of PRIP with LC3; it also increased the cellular colocalization of GFP-LC3 and DsRed-PRIP1 in MEFs. Exogenous overexpression of PRIP in *PRIP*-KO MEFs significantly inhibited GFP-dot formation. Furthermore, starvation increased the formation of GFP-positive dots in *PRIP*-KO cells. These results suggest that PRIP is a negative modulator of autophagy. Consistent with this, we did not see many dots in MEFs overexpressing DsRed-PRIP (Fig. 2B, right lower panel). In addition, the enhanced colocalization of PRIP1 and LC3 in the cytosol suggests that PRIP negatively regulates the early stage of autophagosome formation.

To confirm that PRIP is involved in the early stage of autophagosome formation, we treated WT and *PRIP*-KO cells with LY294002 or wortmannin, that is, inhibitors of class III phosphatidylinositol 3-kinase, which is required for autophagosome formation. When the reagent-treated cells were starved, GFP-LC3 puncta did not form in *PRIP*-KO cells as they did in WT cells (data not shown), indicating that the newly formed GFP-positive dots are autophagosomes. Autophagy is inhibited by the mammalian target of rapamycin (mTOR), a central cell growth regulator that integrates growth factor and nutrient signals; phosphorylation of mTOR is an index of autophagy activity [31]. We then tested the mTOR phosphorylation level in MEFs by using a phosphospecific antibody; no clear difference was observed between the genotypes in the nutrient-rich or starvation conditions (data not shown). PRIP was not degraded during starvation-induced autophagy, although PRIP was colocalized with GFP-LC3 positive dots, indicating that PRIP is not an autophagy substrate, unlike LC3 and p62 [28]. These data indicate that PRIP appears to participate in the autophagy process during autophagosome formation, but not in the upstream signaling pathways regulated by mTOR or phosphatidylinositol 3-kinase.

Here, we used GFP-LC3 to monitor autophagosome formation and found that starvation increased the colocalization between PRIP and GFP-LC3, and PRIP negatively regulated LC3-promoted autophagy process. However, the molecular mechanisms of this negative modulation are not yet defined. Furthermore, PRIP bound to GABARAP [15], which is implicated in later stages of the formation of the autophagosome (autophagosome maturation) [32]. We observed a slight increase in the interaction between PRIP and GABARAP (especially form II) in response to nutrient starvation (data not shown). Thus, PRIP may affect GABARAP-induced autophagic activity. Further studies on the function of PRIP in the regulation of autophagosome formation could improve our understanding of the complicated mechanisms that regulate autophagy and, in turn, provide insights into related human diseases.

Acknowledgments

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The authors declare that they have no competing interests.

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