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Identification of glucose-6-phosphate transporter as a key regulator functioning at the autophagy initiation step

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ABSTRACT

Autophagy is a catabolic process involving autophagosome formation via lysosome. However, the initiation step of autophagy is largely unknown. We found an interaction between ULK1 and ATG9 in mammalian cells and utilized the interaction to identify novel regulators of autophagy upstream of ULK1. We established a cell-based screening assay employing bimolecular fluorescence complementation. By performing gain-of-function screening, we identified G6PT as an autophagy activator. G6PT enhanced the interaction between N-terminal Venus-tagged ULK1 and C-terminal Venus-tagged ATG9, and increased autophagic flux independent of its transport activity. G6PT negatively regulated mTORC1 activity, demonstrating that G6PT functions upstream of mTORC1 in stimulating autophagy.

1. Introduction

Macroautophagy, autophagy hereafter, is an intracellular degradation system and is initiated by the formation of a double-membrane vesicle, called an autophagosome, which fuses with lysosomes to degrade cytoplasmic components [1]. Autophagy degrades subcellular organelles such as mitochondria and large protein aggregates [2]. Since autophagy contributes to cell growth, adaptation, and differentiation, its malfunction is intimately associated with human diseases [3–5]. Autophagy is activated under nutrient deprivation to maintain cellular metabolic homeostasis [6]. Amino acid starvation induces autophagy by inhibiting mTORC1, which is under the modulation of Rag GTPases [7]. On the other hand, mTORC1 inhibits autophagy by phosphorylating ULK1 [8] and AMBRA, an E3 ligase, to ubiquitinate ULK1 for degradation [9]. During glucose starvation, AMPK activates ULK1 and TSC2 or inhibits Raptor [10,11]. Autophagy is also regulated by cellular stresses. ER stress affects starvation-induced autophagy through eIF2α [12,13]. However, prolonged ER stress impairs autophagy via IRE1 [14]. During hypoxia, HIF1α increases BNIP3 and BNIP3L (NIX) to initiate autophagy [15], and PERK increases LC3 and ATG5 to control autophagosome formation [16]. In line with the identification of new autophagy signals, elucidation of signal modulators is necessary for a better understanding of the autophagy mechanism.

To date, about 30 autophagy-related genes (ATGs) have been reported to be required for autophagosome biogenesis [17]. Among them, ULK1 is required for the initiation step of autophagy. ULK1, a serine/threonine kinase, forms complexes with FIP200, ATG13, and ATG101 [18,19], and the complexes are mainly controlled by mTORC 1 [8,19] and AMPK [11]. The kinase activity of ULK1 is required for the recruitment of VPS34 to isolation membrane [20,21]. Unlike other ATG proteins, ATG9 is a membrane protein containing six trans-membrane domains. ATG9 shuttles

Abbreviations: BiFC, bimolecular fluorescence complementation; CHA, chlorogenic acid; G6PT, glucose-6-phosphate transporter; VC, C-terminus of Venus vector; VN, N-terminus of Venus vector

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between autophagosomes and the *trans* Golgi network during autophagosome biogenesis to deliver membrane source to autophagosomes [22]. The movement of ATG9 is dependent on the activity of the ULK1 with class III PI3K complex. Moreover, like ATG16L1, ATG9 functions to traffic vesicles derived from the plasma membrane [23]. In yeast, Atg1, an ULK1 homolog, interacts with Atg9 through Atg17, a counterpart of FIP200, and the interaction is increased by autophagy activation [24]. A recent study also reported an interaction between Atg1 and Atg9 in *Drosophila* during starvation-induced autophagy [25]. However, this interaction has not been revealed in higher eukaryotes.

Glucose-6-phosphate transporter (G6PT) is embedded in the ER membrane by 10 transmembrane domains [26]. It functions to transport cytosolic glucose-6-phosphate (G6P) into the ER lumen during gluconeogenesis or glycogenolysis by complexing with glucose-6-phosphatase (G6Pase)-α (in the liver, kidney, and intestine) or -P (in neutrophils). Accordingly, deficiency of G6PT/G6Pase complex causes glycogen storage disease (GSD), leading to the homeostatic imbalance of glucose [27]. To identify regulators of the initiation step of autophagy, we performed cell-based functional screening. We identified G6PT as an autophagy activator and hypothesized that it regulates autophagy through mTORC1.

2. Materials and methods

2.1. DNA Constructs

ULK1, ATG9 and G6PT cDNAs were subcloned into *BglII*/*XbaI* of pFLAG-VN173, ApnI of pHA-VC155, and *HindIII* site of pcDNA3-HA (Invitrogen). Bimolecular fluorescence complementation (BiFC) assay was performed as described [28]. G6PT shRNAs were constructed using forward and reverse synthetic 19-base sequences (#1, 5′-1243CGA AAC ATC CGC ACC AAG A-3′; #2, 5′-$_{107}$CAT CAT TGG AAG AGA T-3′), which were cloned into *BglIUHindIII* sites of pSuper-neo (Oligoengine). The pHTTex120-GFP, which is a fragment of Huntingtin (HTT) exon1 containing expanded polyglutamine (n = 120) fused to GFP, was described [14].

2.2. Cell culture and DNA transfection

HEK293T and m5-7 cells were cultured in DMEM and Hep3B cells in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone) at 37 °C under an atmosphere of 5% CO_2. Transfection was carried out using Polyfect reagent (Qiagen), according to the manufacturer's instructions.

2.3. Site-directed mutagenesis

G6PT (R28H, G149E, A373D) mutants were generated using a Quickchange Site-Directed Mutagenesis kit (Stratagene) and synthetic oligonucleotides containing mutations in the corresponding positions.

2.4. Generation of stable cell lines

Hep3B cells were transfected with pcDNA3-HA, pG6PT-HA, or pG6PT shRNA for 24 h and grown in the media containing 1 mg/mL G418 sulfate (Invitrogen) for 2 weeks. Then, single clones of stable cells were isolated using the standard selection method.

2.5. Antibodies and Western blot analysis

Cell extracts were prepared and analyzed as previously described [14]. Immunoreactive proteins were visualized using an enhanced chemiluminescence method. The following antibodies were used for Western blot analysis: LC3, ATG5, Beclin1 (Novus), ATG9A (Novus, Cell signaling), pS6K, S6K, pAMPK, AMPK, p4E-BP1, 4E-BP1, pULK1 (Cell signaling), FLAG (Sigma), p62 (Abnova), actin, ULK1, GFP (Santa Cruz Biotechnology), and G6PT (Abcam).

2.6. Immunoprecipitation assay

Cells were lyzed in RIPA buffer (50 mM Tris–Cl pH 7.5, 50 mM NaCl, 1% Triton X-100, 0.2% SDS, 1 mM EDTA, and 1 mM PMSF). After clarifying by centrifugation, cell lysates were incubated with anti-ULK1 antibody or FLAG (M2) bead (Sigma) for 12 h at 4 °C. After adding protein A/G-Bead (Santa Cruz Biotechnology), the mixtures were incubated for additional 6 h at 4 °C. The immunocomplexes were washed five times with PBS, solubilized with sample buffer lacking β-mercaptoethanol, and detected by Western blotting.

2.7. Immunostaining

Cells grown on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA. After incubation with antibody and Hoechst 33258, cells were visualized under an LSM confocal fluorescence microscope (Zeiss).

2.8. RT-PCR

Total RNA was purified using Trizol reagent (Ambion) according to the manufacturer's instructions. The following primer sets were used for PCR: *p62*, forward 5′-ATG GCC ATG TCC TAC GTG AAG GAT G-3′ and reverse 5′-AGA GGG CTA AGG GCA GCT GCC ACA C-3′; *β-actin*, forward 5′-GAG CTG CCT GAC GGC CAG G-3′ and reverse 5′-CAT CTG GAA GGT GGA C-3′.

2.9. Statistical analysis

Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, or Student's t-test, as appropriate. Error bars represent S.D. or S.E.M., as indicated. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Establishment of the ULK1-VN/ATG9-VC BiFC assay to screen for autophagy activators

Based on the previous reports showing that Atg1 interacts with Atg9 in *Drosophila* and yeast [24,25], we examined whether ULK1 interacts with ATG9 in mammalian cells. Data from the immunoprecipitation assay revealed that ATG9-FLAG interacted with HA-ULK1 in the transfected cells (Fig. 1A and B). We further found that endogenous ATG9 was immunoprecipitated together with ULK1 (Fig. 1C), indicating that ULK1 interacts with ATG9 in mammalian cells. These results led us to establish a screening assay to identify autophagy modulators. Since the BiFC assay is a tool to observe protein–protein interactions in living cells, we adopted this method to identify novel proteins that regulate the initiation step of autophagy around ULK1.

To establish the BiFC assay, ULK1 was fused to the N-terminus of the Venus vector (ULK-VN), while ATG9 was fused to the C-terminus of the vector (ATG9-VC) (Fig. S1A). In the immunoprecipitation assay, we detected an interaction between ULK1-VN and ATG9-VC, which was apparently increased under serum starvation (Fig. 1D). Western blot analysis also revealed that serum starvation following ectopic expression of both ULK1-VN and ATG9-VC