Biochemical and Biophysical Research Communications 456 (2015) 275–281

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Trans-Golgi protein p230/golgin-245 is involved in phagophore formation

Miwa Sohda [a], Yoshio Misumi [b,*], Shigenori Ogata [c], Shotaro Sakisaka [d], Shinichi Hirose [e], Yukio Ikehara [f], Kimimitsu Oda [a]

[a] Division of Oral Biochemistry, Niigata University Graduate School of Medical and Dental Sciences, Chuo-ku, Niigata 951-8514, Japan
[b] Department of Cell Biology, Fukuoka University School of Medicine, Jonan-ku, Fukuoka 814-0180, Japan
[c] Joint Laboratory for Frontier Medical Science, Fukuoka University School of Medicine, Jonan-ku, Fukuoka 814-0180, Japan
[d] Department of Internal Medicine, Fukuoka University School of Medicine, Jonan-ku, Fukuoka 814-0180, Japan
[e] Department of Pediatrics, Fukuoka University School of Medicine, Jonan-ku, Fukuoka 814-0180, Japan
[f] Fukuoka Tenjin Medical Rehabilitation Academy, Chuo-ku, Fukuoka 810-0001, Japan

ARTICLE INFO

Article history:
Received 7 November 2014
Available online 28 November 2014

Keywords:
Autophagy
Golgi
Immunochemistry
Membrane trafficking
siRNA

ABSTRACT

p230/golgin-245 is a *trans*-Golgi coiled-coil protein that is known to participate in regulatory transport from the *trans*-Golgi network (TGN) to the cell surface. We investigated the role of p230 and its interacting protein, microtubule actin crosslinking protein 1 (MACF1), in amino acid starvation-induced membrane transport. p230 or MACF1 knock-down (KD) cells failed to increase the autophagic flow rate and the number of microtubule-associated protein 1 light chain 3 (LC3)-positive puncta under starvation conditions. Loss of p230 or MACF1 impaired mAtg9 recruitment to peripheral phagophores from the TGN, which was observed in the early step of autophagosome formation. Overexpression of the p230-binding domain of MACF1 resulted in the inhibition of mAtg9 trafficking in starvation conditions as in p230-KD or MACF1-KD cells. These results indicate that p230 and MACF1 cooperatively play an important role in the formation of phagophore through starvation-induced transport of mAtg9-containing membranes from the TGN. In addition, p230 itself was detected in autophagosomes/autolysosome with p62 or LC3 during autophagosome biogenesis. Thus, p230 is an important molecule in phagophore formation, although it remains unclear whether p230 has any role in late steps of autophagy.

1. Introduction

Macroautophagy (simply referred to as autophagy) is a ubiquitous eukaryotic system for the turnover of cytoplasmic materials in a lysosome-dependent manner, and is carried out by evolutionarily conserved Atg (autophagy related) factors. The autophagy process is initiated by the formation of the isolation membrane (phagophore). Then, the isolation membrane expands and closes to become a double membrane vesicle called the autophagosome [1]. It has been proposed that the autophagosome membrane originates from a number of sources, including the endoplasmic reticulum (ER), the Golgi apparatus, mitochondria, and the plasma membrane (PM) [2].

It is well accepted that the Golgi apparatus is one of the membrane sources of the phagophore, and that the existence of the vesicle fusion process is essential at the early step of autophagosome formation [3,4]. In fact, pre- and post-Golgi vesicle trafficking machineries, such as SNAREs, Rabs, and tethering factors, are required for the expansion of the phagophore [3]. The multi-spanning transmembrane protein Atg9 has been supposed to play an important role for the phagophore membrane biogenesis [4]. In yeast, during starvation, Atg9 vesicles derived from the Golgi are assembled into a preautophagosomal structure, and then membranes derived from Atg9 vesicles is incorporated into the phagophores [5]. Mammalian Atg9, known as mAtg9 or Atg9a, is localized at the *trans*-Golgi network (TGN) and at endosomes in feeding cells, and cycles among the endosomes, the TGN and phagophores/autophagosomes during starvation conditions [4]. Although the regulatory mechanism of mAtg9 trafficking has been studied [6–8], the precise role and transport mechanism of membranes with mAtg9 from the Golgi for autophagosome biogenesis is not sufficiently understood. Recently, it was demonstrated that

Abbreviations: AP2, adaptor-protein 2; EBSS, Earle's balanced salt solution; ER, endoplasmic reticulum; KD, knock-down; MACF1, microtubule actin crosslinking protein 1; LC3, microtubule-associated protein 1 light chain 3; PM, plasma membrane; TGN, *trans*-Golgi network.

* Corresponding author.
E-mail address: misumi@fukuoka-u.ac.jp (Y. Misumi).

http://dx.doi.org/10.1016/j.bbrc.2014.11.071
0006-291X/

AP2/clathrin-dependent endocytosis plays an important role in the transport of mAtg9 from the PM to the phagophores, suggesting that mAtg9 trafficking from the TGN to the PM is important in autophagosome biogenesis [9].

The TGN coiled-coil protein p230 (also known as golgin-245 or t-golgin-1) is a GRIP domain-containing protein (GRIP-golgin), and is peripherally associated with the cytoplasmic face of the TGN membrane [10]. p230 is required for the secretion of TNF induced with LPS in mouse macrophages [11] and is involved in HLA class I surface expression induced by INF-γ [12]. Kakinuma et al. [13] reported that p230 interacts with a protein that crosslinks microtubules to the actin cytoskeleton microfilament1 (MACF1), and that the disruption of this interaction inhibits the transport of the p230-specific cargo, GPI-anchored protein from the TGN to PM. These results suggest that p230 plays a role in the regulative transport of specific proteins from the TGN to the PM.

In this study, we investigated the role of p230 and its interacting protein, microtubule actin crosslinking protein 1 (MACF1), on amino acid starvation-induced membrane transport. p230 participation in autophagy was examined using p230 knock-down (KD) cells, and revealed that p230 and its interacting protein MACF1 cooperate in the early step of autophagy involving the transport of mAtg9. In addition, p230 itself is recruited to peripheral structures and the PM with mAtg9 in response to starvation, and is detected in the phagophores/autophagosomes with p62 or microtubule-associated protein 1 light chain 3 (LC3) during autophagosome biogenesis.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies used were as follows: mouse monoclonal anti-golgin-97 (Life technologies Inc., Rockville, MD), anti-p62 and anti-p230 (BD Bioscience, San Diego, CA), anti-LC3 (M152-3 and M186-3; MBL, Nagoya, Japan), and anti-β-actin (clone AC-15; Sigma–Aldrich, St. Louis, MO); rabbit monoclonal anti-mAtg9 (Abcam, Cambridge, UK); rabbit polyclonal anti-ACF7/MACF1 (Merck Millipore, Darmstadt Germany), anti-AP2B1 (Proteintech Group Inc., Chicago, IL), anti-LC3 (PD036), anti-p62 (PM045) (MBL), and anti-Arl1 (a gift from Dr. Kazuhisa Nakayama); and AlexaFluor^{488}-conjugated goat anti-rabbit, or anti-mouse IgG, and AlexaFluor^{546}-conjugated goat anti-rabbit or goat anti-mouse IgG (Life Technologies Inc.). E64d and pepstatin A were purchased from the Peptide Institute Inc. (Osaka, Japan), and dynasore was from Sigma–Aldrich.

2.2. Plasmid construction and transfection

cDNA fragments of MACF1 were obtained by PCR of HeLa cell oligo dT-primed cDNA as the template using the DNA primer 5′AGAATTCTTAGCACTGGAGCGGCAAAGGAAACTG3′ and 5′AGTCGACTCATCGATACGCATCCTTGTTGGGATG3′ for the p230-binding domain (amino acid number 5044–5156) or 5′AGAATTCGATACCAGCAATAGTTCTTCCCCG3′ and 5′ACTCGAGTTATCGCTTGGGACCTGGAGTCC3′ for the non-specific domain (amino acid number 5328–5430). DNA fragments were subcloned into the pSG5 expression vector, followed by attachment with an HA-tag sequence. Cells were transfected using Fugene 6 transfection reagent (Promega Corp., Madison, WI) and incubated for 24 h.

2.3. Cell culture and siRNA treatment

HeLa cells were maintained in Eagle's MEM with nonessential amino acids and 10% FCS. RNA interference was performed in HeLa cells using Lipofectamine RNAiMAX (Life Technologies Inc.) with ON-TARGETplus small interfering RNA (siRNA) (Thermo Fisher Scientific, Waltham, MA) for p230 and Stealth siRNA for MACF1 (Life Technologies Inc., HSS146438). For p230-KD experiments, one day after treatment, cells were re-treated with siRNA oligos for an additional day. p230 was tagged with the sequence GUAGAUGACUGGUCCAAUA and the nonspecific control duplex was used as a control. For MACF1-KD experiments, one day after treatment, cells were re-treated with siRNA oligos for an additional day.

2.4. Cell lysate preparation and immunoprecipitation

Cells were harvested and lysed in lysis buffer as described previously [14]. For immunoprecipitation, cells were lysed in 1% TX-100 lysis buffer. The resultant lysate was subjected to immunoprecipitation using protein A-Sepharose as described previously [14].

2.5. SDS–PAGE and immunoblotting

Cell lysates and immunoprecipitates were analyzed by SDS–PAGE (4%, 7% or 15% gels) and immunoblotting with the indicated antibodies. The immunoreactive proteins were visualized using an ECL-Prime kit or ECL-Select kit (GE Healthcare, Piscataway, NJ) by a LAS4000 imaging system (Fujifilm, Tokyo, Japan).

2.6. Immunofluorescence staining and fluorescence microscopy

After the desired incubation times, cells were fixed and immunostained as previously described [14]. Stained cells were observed with an LSM PASCAL confocal microscope with a ×63 objective (Carl Zeiss, Jena, Germany). Confocal images are presented as sections of 0.4 μm in thickness.

3. Results and discussion

3.1. The depletion of p230 or its binding partner MACF1 inhibits autophagy

To explore roles of p230 and MACF1 on autophagy, p230-KD or MACF1-KD HeLa cells were investigated. The immunoblotting analysis showed that the each protein was knocked down efficiently, whereas the amounts of MACF1 and p230 in p230-KD and MACF1-KD cells, respectively, were almost the same as those in control siRNA-treated cells (Fig. 1A). We monitored the autophagic flux in p230-KD and MACF-KD cells. After a 2 h incubation in Earle's balanced salt solution (EBSS), with or without lysosomal protease inhibitors (E64d and pepstatin A, 10 μM each), cells were subjected to immunoblotting for LC3 to monitor the conversion from LC3-I to LC3-II [15]. LC3-II was significantly accumulated in control cells with protease inhibitors in starvation conditions as compared with that in nutrient-rich conditions. In contrast, the accumulation of LC3-II was not observed in either feeding or starvation conditions in p230-KD and MACF1-KD cells (Supplementary Fig. S1). About a 1.9-fold increase of autophagic flux was observed in control cells under starvation compared with under feeding, although a lower level of autophagic flux was found in both in p230-KD and MACF1-KD cells (Fig. 1B). To ensure the effect of depletion of p230 and MACF1 on autophagy, we performed fluorescence microscopy assays of LC3-positive puncta. Upon starvation, many LC3-positive puncta were observed in control cells, whereas fewer ones were found in p230 or MACF1 siRNA-treated cells (Supplementary Fig. S2). Upon starvation, the number of LC3 puncta per cell increased only 1–1.5-fold in KD cells, in contrast to the 8-fold increase in control cells (Fig. 1C). This suggests that