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Molecular determinants that mediate the sorting of human ATG9A from the endoplasmic reticulum

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ABSTRACT

ATG9A is a multispanning membrane protein required for autophagosome formation. Under basal conditions, neosynthesized ATG9A proteins travel to the Golgi apparatus and cycle between the *trans*-Golgi network and endosomes. In the present work, we searched for molecular determinants involved in the subcellular trafficking of human ATG9A in HeLa cells using sequential deletions and point mutations. Deletion of amino acids L^{340} to L^{354} resulted in the retention of ATG9A in the endoplasmic reticulum. In addition, we found that substitution of the $L^{711}YM^{713}$ sequence (located in the C-terminal region of ATG9A) by alanine residues severely impaired its transport through the Golgi apparatus. This defect could be corrected by oligomerization of the mutant protein with co-transfected wild-type ATG9A, suggesting that ATG9A oligomerization may help its sorting through biosynthetic compartments. Lastly, the study of the consequences of the LYM/AAA mutation on the intracellular trafficking of ATG9A highlighted that some newly synthesized ATG9A can bypass the Golgi apparatus to reach the plasma membrane. Taken together, these findings provide new insights into the intracellular pathways followed by ATG9A to reach different subcellular compartments, and into the intramolecular determinants that drive the sorting of this protein.

1. Introduction

Macroautophagy is a highly conserved organelle and protein degradation mechanism that is upregulated in response to nutrient deprivation. It begins with the formation of an isolation membrane, known as phagophore, which elongates and progressively surrounds portions of cytoplasm. Although the origin of this membrane is still unclear, some data suggest that it could derive from different compartments: the Golgi apparatus, the endoplasmic reticulum (ER), the plasma membrane, recycling endosomes and even mitochondria [1]. The phagophore then closes to form a vesicle called autophagosome, which will fuse with the vacuole in yeast, or with lysosomes in mammalian cells, allowing degradation of sequestered contents. The different steps of the autophagy process are regulated by approximately 30 proteins belonging to the autophagy-related (ATG) family. ATG9 is the only transmembrane protein of this family, and it has been proposed that this protein participates in the recruitment of membranes during autophagy initiation.

The knockdown of *Atg9* in yeast [2,3] or of its homolog *ATG9A* in mammalian cells [4–6] significantly reduces autophagosome formation. It has been documented that, under basal conditions, yeast Atg9 cycles between the PreAutophagosomal Structure or Phagophore Assembly Site (PAS) and several peripheral punctuate structures, some of them positive for mitochondrial markers [7,8]. Other studies conducted in yeast suggest that small cytoplasmic vesicles enriched in Atg9 may originate from the Golgi apparatus [9,10]. After induction of autophagy, Atg9 molecules, possibly self-associated [11], are recruited to the PAS from where they become part of the autophagosomal membrane. Once the autophagosome is fully formed, Atg9 is recycled back to the PAS [10,12]. In mammalian cells, ATG9A cycles between the *trans*-Golgi network (TGN) and endocytic compartments [5], where it co-localizes with early, recycling and late endosomal markers [5,6]. After nutrient-starvation, the juxta-nuclear/TGN localization of ATG9A is lost while its co-localization with endosomal and autophagosomal markers increases [5]. Additionally, ATG9A has been detected on the plasma membrane from where it can be internalized and directed to recycling endosomes via clathrin-mediated endocytosis [13–15].

Mammalian ATG9A possesses six transmembrane domains with cytosolic NH_2- and COOH-terminal tails [5]. This protein also contains one used N-glycosylation site located in its first luminal loop, which acquires a complex-type oligosaccharidic chain [5]. This post-translational modification is indicative of the transport of newly synthesized ATG9A through the Golgi apparatus where high-mannose glycans are processed into complex-type oligosaccharides. However, little is known about the molecular mechanisms that mediate the sorting of ATG9A out of the ER in mammalian cells. Packaging of newly synthesized and

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correctly folded transmembrane proteins into ER-derived COPII vesicles that travel to the Golgi can be triggered by the recognition of specific amino acid sequences by adaptor proteins at ER exit sites (ERES) [16]. Once in the ERGIC (ER-Golgi intermediate compartment), or upon arrival at the *cis*-Golgi compartment, the proteins can either proceed through the *medial* and *trans*-Golgi compartments, or be recycled back to the ER [17]. In this study, our objective was to search for intramolecular determinants that mediate the biosynthetic transport of human ATG9A. Interestingly, in addition to providing a better understanding of the sorting of ATG9A by the "classical" ER to Golgi pathway, our findings highlighted that this is not the only route by which the protein can travel to the plasma membrane and endosomes.

2. Material and methods

2.1. Material

Unless otherwise specified, chemicals were obtained from Sigma-Aldrich, as well as mouse *anti*-GAPDH, mouse *anti*-Tubulin, mouse *anti*-AP-1 (100/3) and rabbit *anti*-Hemagglutinin (HA) antibodies. Mouse and rabbit *anti*-MYC antibodies were bought from Cell Signaling and rabbit *anti*-ATG9A from Novus Biologicals. The epitope recognized by the latter is localized in the C-terminal region of ATG9A, between residues 750–839. Rabbit *anti*-VPS26 and *anti*-Calnexin were obtained from Abcam, sheep *anti*-SEZ6L2 from R&D Systems, mouse *anti*-GM130 from BD Biosciences and mouse *anti*-GOLGIN97 from Santa Cruz. Mouse *anti*-ERGIC53 and rabbit *anti*-SEC61β antibodies were provided by Xavier De Bolle's group (URBM, University of Namur) and Bernhard Dobberstein's group (ZMBH, Heidelberg), respectively. HRP (Horseradish Peroxidase)-coupled and Alexa Fluor™-coupled secondary antibodies were obtained from DAKO and Life Technologies, respectively. Cell culture media, trypsin and antibiotics were obtained from LONZA, and fetal bovine serum from Sigma.

2.2. DNA constructs

The sequence coding for human ATG9A-MYC-FLAG (obtained from OriGene, RC222513, NM_024085) has been inserted via EcoRI and SacII in a pcDNA3.1 (+) plasmid previously modified in order to insert a SacII restriction site in its multiple cloning site, using classical molecular biology methods.

The following mutants were generated using PCR to introduce the mutation followed by cloning using either restriction/ligation or InFusion™ HD Cloning methods (Clontech) following the manufacturer's instructions: ATG9A-N99D, ATG9A-LYM/AAA (L711A-Y712A-M713A), ATG9A-ΔL593-S761, ATG9A-ΔS592-A682, ATG9A-ΔY701-A760, ATG9A-ΔY701-Q720, ATG9A-ΔA721-G739, ATG9A-ΔE740-A760, ATG9A-ΔL340-L354.

HA-tagged constructs of ATG9A (wild-type or mutated) were obtained by insertion of a linker coding for HA and a STOP codon via the NotI restriction site located at the C-terminal end of ATG9A in pcDNA3.1(+).

The procathepsin D-KDEL-MYC construct has been described in Boonen et al. [18].

2.3. Cell culture and transient transfection

HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere with 5% CO_2. Transient transfections of plasmids were performed using XtremeGENE9 reagent (Roche), following the manufacturer's instructions. Transfection media were removed after 8 h and the cells were lysed in PBS-TX100 1% containing protease inhibitors (Roche) 16 h later (i.e. 24 h post-transfection). When indicated, the cells were treated with 50 μM of leupeptin/50 μM of pepstatin A, or with 5 μM of MG132 during this 16 h period.

2.4. Metabolic labelling and immunoprecipitation

Metabolic labelling was mainly conducted as described previously [18]. Briefly, HeLa cells were transiently transfected with ATG9A fused to a C-terminal MYC tag. After a 24 h post-transfection, cells were starved for 30 min in methionine (Met)/cysteine (Cys)- and FBS-free medium, then incubated with ^{35}S-Met/Cys (Easy Tag Express Protein labelling Mix from Perkin Elmer) for 1 h in Met/Cys-free medium, washed and incubated or not for an additional period of 1, 3 or 6 h in unlabelled complete medium containing FBS and, when indicated 1 μg/mL of Brefeldin A (BFA). ATG9A was then immunoprecipitated from cell lysates. Signals were imaged using a Cyclone Plus Phosphor Imager (Perkin-Elmer) and quantified with the OptiQuant™ software.

2.5. Endoglycosidase treatments

Cell lysates or ^{35}S-ATG9A immunoprecipitates were treated with endoglycosidase H or with PNGase F (from New England Biolabs) following the manufacturer's instructions. The "non-treated" samples were mixed with the Endo H buffer and incubated in the same conditions, as were treated samples (i.e. for 1 h at 37 °C).

2.6. Western blotting

Samples were mixed with Laemmli's sample buffer containing dithiothreitol (DTT, final concentration of 100 mM) and resolved by SDS-PAGE (in 8.3 × 8 cm gels) next to a Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standard form Bio-Rad. After transfer on a polyvinylidene fluoride membrane (PVDF, Perkin Elmer), blocking in 10% fat-free milk in PBS-Tween 0.1%, proteins of interest were detected with specific primary antibodies diluted in PBS-Tween 0.1% containing 5% fat-free milk, followed by incubation with HRP-coupled secondary antibodies. Signals were revealed by chemiluminescence (Western Lightning® Plus–ECL, Perkin-Elmer) and quantified with the ImageJ software.

Of note, the apparent molecular masses of the high-mannose and complex-glycan bearing forms of ATG9A were estimated at 92 and 104 kDa, respectively, using a linear regression model (LOG_{10} of the Protein Standard molecular masses plotted against the distance of migration in a 20 × 17 cm gel, $y = -00092\times + 23679$ with $R^2 = 0.96$).

2.7. Co-immunoprecipitation assay

HeLa cells were co-transfected with wild-type and mutated ATG9A constructs and then lysed 24 h post-transfection in IP buffer (0.1 M Tris HCl, pH 8.0, 0.15 M NaCl and 1% Triton TX-100). Cell lysates were incubated overnight with 50 μL of protein G-Agarose beads (Pierce) which were pre-incubated with mouse *anti*-MYC antibody for 4 h at 4 °C. The next day, the beads were washed five times with ice-cold IP buffer and the immunoprecipitated proteins were eluted in Laemmli's sample buffer with DTT by heating for 5 min at 70 °C, and analyzed by Western blotting. As a control of non-specific protein immunoprecipitation, cell lysates were incubated under the same conditions with a non-relevant antibody (mouse IgG, Santa Cruz).

2.8. Preparation of a fraction enriched in clathrin-coated vesicles (CCV) from HeLa cells

Isolation of CCV from transfected HeLa cells has been conducted mostly as described by Hirst et al. [19]. Briefly, after homogenization of seven 9 cm^2 tissue culture dishes in homogenization buffer (100 mM MES, pH 6.5, 0.5 mM $MgCl_2$, 0.2 mM EDTA supplemented