Lipids of autophagic membranes and their role in the recruitment of Syntaxin 17 to the autophagosomes

Summary of PhD Thesis

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INTRODUCTION

The term "Autophagy" was coined by Christian De Duve, a Belgian cytologist and biochemist in 1963. Autophagy is a natural and comprehensive degradation process that is conserved among eukaryotes. It helps to dispose of obsolete cytoplasmic components such as damaged organelles, aggregated proteins, lipids, invading bacteria etc. to lysosomes to form autolysosomes or to endosomes to form the amphisomes. Eventually, the amphisomes fuse with lysosomes for their degradation and recycling processes. This pathway acts as a survival mechanism, particularly at times of cellular stress, such as nutrient deprivation, oxidative stress, or infection. By recycling cellular components, autophagy provides the necessary building blocks and energy to sustain cellular functions during periods of limited resources. The autophagy pathway is not only essential for cellular survival but also plays a role in various physiological processes, including cellular homeostasis, development, immunity, and aging. Misregulation of autophagy has been implicated in various diseases, such as neurodegenerative disorders,

cancer, and metabolic conditions. Therefore, understanding the molecular mechanisms that govern autophagy has become vital in biomedical research, with the hope of developing therapeutic interventions that can modulate autophagy for the treatment of various diseases. My PhD study, mainly focused on 'Macroautophagy' (hereafter mentioned as only autophagy). The autophagy pathway mainly includes five steps: initiation, elongation, maturation, fusion, and degradation. This pathway initiates with the nucleation of a cup-shaped isolation membrane surrounding cytoplasmic cargo called "phagophore", eventually, it can mature into a double membrane layered vesicle called an "autophagosome". As the final step, the outer autophagosomal membranes fuse with the lysosomes to form "autolysosomes" for the sequestered cargo degradation by lysosomal acidic hydrolases and recycling. The fusion step of autophagosomes and lysosomes is driven mainly by SNARE proteins namely, autophagosomal SNAREs – Syntaxin 17 (STX17 (human) /Syx17 (*Drosophila*)) and/or YKT6, cytosolic SNARE–SNAP29, and lysosomal SNARE–VAMP7/VAMP8 (Vesicle-associated membrane

protein7/8) and by multiple tethering factors such as homotypic fusion and protein sorting complex (HOPS), pleckstrin homology domain-containing family member 1 (PLEKHM1), and Ectopic P-granules autophagy protein 5 homolog (EPG5).

The main focus of this study was on the autophagosomal SNARE protein called Syntaxin 17. Our research group and others have demonstrated the significant role of SNARE Syntaxin 17 during the fusion of autophagosomes and lysosomes. Depletion of Syx17 using RNAi in Drosophila larvae caused a characteristic phenotype where Atg8a positive autophagosomes accumulated in the perinuclear region of fat body cells due to lack of autophagosome-lysosome fusion thus leading to a complete block of autophagy degradation. The similar fusion defects are also observed in mammals and Caenorhabditis elegans suggesting an evolutionarily conserved role of autophagosomal SNARE Syntaxin 17 during autophagy.

This conserved pathway relies on the de novo synthesis of double-membrane vesicles. Therefore, lipids play a crucial role in various aspects of autophagy, contributing

to the formation of autophagosomes and serving as a source of energy during this process. The autophagy pathway involves a core set of autophagy-related proteins (ATG proteins), so far 40 ATG proteins have been identified and extensively studied. Still, many questions remain unanswered in this emerging field. One among them is the "role of lipids during autophagy". Our research focuses on identifying the specific lipid molecules that interact with Syntaxin 17 (from both *Drosophila* and *Homo sapiens*) and facilitating its incorporation into the autophagosomal membrane, with a combination of *in vitro* and *in vivo* approaches. To perform these lipid-protein interaction studies it is important to know the lipids composing autophagic structures. Surprisingly, no research has been held to unravel the lipid profile of autophagic structures especially from *Drosophila*.

AIMS OF THE THESIS

Autophagy is spatiotemporally governed by a series of membrane-associated proteins, therefore it is essential to understand the interactions between membrane lipids and

autophagy proteins. While several autophagy proteins that interact with autophagic membranes have been studied, there is no information available on the lipids involved in the recruitment of Syntaxin 17 to the autophagosomes. During the autophagosome-lysosome fusion step, Syntaxin 17 translocate from the cytosol to autophagosomes but not to the phagophores. However, the mechanism underlying the transient visit of Syntaxin 17 to the autophagosomes is not yet understood. One possibility is that de novo generated signaling lipids on the membrane, such as phosphoinositides especially, PI(3)P, PI(4)P, or PI(3,5)P_2, can be the fundamental signal to recruit Syntaxin 17 to the matured autophagosomes. As known, lipids are not only for compartmentalization and energy storage but also function as relevant regulators in cellular signaling. Therefore we speculate that lipids may have a role in the recruitment of Syntaxin 17 to the autophagosomes during the fusion step.

Thus, our research aims briefly are the following:

✓ Investigate the role of lipids in facilitating the autophagosomal recruitment of Syntaxin 17 (*Drosophila* and *Homo sapiens*) during autophagy *in vitro* and *in vivo*

- Establish a method for the isolation of intact autophagic vesicles from the *Drosophila*
- Decipher the lipid profiles of isolated autophagic structures

✓ Study the specific structural features of Syntaxin 17 that mediate lipid binding

✓ Confirm the significance of amino acids responsible for lipid-protein interactions.

METHODS

- Isolation of autophagosomes by immunoprecipitation
- Immunoblot and Antibodies
- Fluorescent Microscopy for validation of the autophagic structures
- Atomic Force Microscopy (AFM)
- Lipidome analysis for the lipids extracted from autophagic membranes
- Liposome flotation assay
- Cell culture, plasmids, and transfection

- PI(4)P immunostaining
- Microscopy
- Structural models
- Molecular dynamic simulations

SUMMARY OF FINDINGS

In our study, we successfully developed a method for immunoprecipitating autophagic membranes from both starved control and Atg2^{-} *Drosophila* adult flies, confirming the integrity of the isolated vesicles through western blotting, fluorescent microscopy, atomic force microscopy, and by screening with various organelle markers to ensure a high degree of purity. Eventually, we characterized the phospholipid composition of these autophagic structures using mass spectrometry, revealing a distinct lipid profile. Briefly, the phospholipid profile of autophagic structures isolated from the control is following: PE (67 %) + PC (15 %) + PI (10 %) + PA (5 %) + PS (3 %) and for the Atg2^{-} samples: PI (40 %) + PA (21 %) + PS (15 %) + PE (14 %) + PC (10 %). While shorter fatty acyl chains are dominant in the control autophagic membranes, the longer fatty acyl chain lengths

are abundant in Atg2^{-}. Although the degree of unsaturation was higher in the membranes isolated from the control flies (63.4 %), it was even higher in the Atg2^{-} samples (86 %). The striking differences in the lipid patterns reflect the potential role of Atg2 lipid transporter protein and also the lipid dynamic characteristics causing remodeling effects during phagophores maturation and closure of autophagosomes. The autophagosomes have specific requirements for membrane curvature and fusion ability which are determined by unique phospholipid composition.

Characterisation of the lipid composition of autophagic membranes from the control flies supported the understanding of lipid-protein interactions during autophagy by focusing on the recruitment of Syntaxin 17 to the autophagosomes. In line with this, further *in vitro* reconstitution experiments were performed using recombinant *Drosophila* Syntaxin 17 (Syx17) and human Syntaxin 17 (STX17) proteins and generated liposomes of 100 nm in diameter. Liposome flotation assay was the most reliable biochemical method to study the lipid-protein interactions used in this study. Initial experiments

demonstrated that Syntaxin 17 could directly associate with *in vitro* autophagosome mimetics without requiring additional cofactors, later showing a preference for negatively charged membranes, particularly phosphatidylinositols (PIPs) like PI(3)P, PI(4)P, and PI(3,5)P_2. This specificity suggests a fundamental role of negatively charged lipids in the recruitment of STX17/Syx17 to autophagosomes. Additional investigations highlighted that STX17's recruitment is influenced not just by the membrane's negative charge but also by its lipid composition. Interestingly, STX17's integration was deep into liposomes and its recruitment to autophagosomes is independent of membrane curvature. Our data from HEK-293 cells, using specific fluorescent reporters, indicated a significant co-localization between STX17 and PI(4)P on autophagosomes, surpassing interactions with PI(3)P and PI(3,5)P_2, with PI(4)P's presence, facilitated by PI4K2A, proving crucial for STX17's autophagosomal recruitment during lysosomal fusion. Molecular simulation and cellular mutagenesis studies pinpointed essential basic amino acids at the C-terminus of Syntaxin 17 necessary for binding PI(4)P on

autophagosomes, a finding further validated through functional assays. These results collectively underscore the pivotal role of electrostatic interactions between the negatively charged PI(4)P on autophagosomes and positively charged amino acids at the C-terminus of Syntaxin 17 in mediating its recruitment during the autophagy fusion step.

PUBLICATIONS

Articles basis for my dissertation:

1. Laczkó-Dobos, H*., Bhattacharjee, A*., **Maddali, AK*.**, Kincses, A., Abu Ammar, H., Sebők-Nagy, K., Páli, T., Dér, A., Hegedűs, T., Csordás, G., Juhász, G. (2024). PI(4)P promotes Syntaxin 17 recruitment to autophagosomes for lysosomal fusion. *Autophagy*. (Q1/D1, IF: 13.3).

   **equal author contributors*.

2. Laczkó-Dobos, H., **Maddali, A. K**., Jipa, A., Bhattacharjee, A., Végh, A. G., & Juhász, G. (2021). Lipid profiles of autophagic structures isolated from wild type and Atg2 mutant *Drosophila*. *Biochimica et Biophysica Acta. Molecular and Cell Biology of Lipids*, *1866*(3). (Q2, IF: 4.8).

Other articles

3. Balázs, V., **Maddali1, AK.**, Nurgul, D., Viktor, V., Imre, MB. (2020). TERT promoter alterations could provide a solution for Peto's paradox in rodents. *Scientific Reports*, *20815*(13). (Q1/D1, IF: 4.6).

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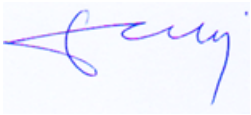
CO-AUTHOR WAIVER

I, Dr. Laczkó-Dobos Hajnalka as the supervisor and contributing author for the published results (Laczkó-Dobos, H., *et al*, Autophagy 2024, PI(4)P promotes Syntaxin 17 recruitment to autophagosomes for lysosomal fusion, and Laczkó-Dobos, H., *et al*, Biochimica et Biophysica Acta. Molecular and Cell Biology of Lipids, 1866(3), Lipid profiles of autophagic structures isolated from wild type and Atg2 mutant *Drosophila*.), hereby certify that I am familiar with the thesis of the Ph.D candidate Maddali Asha Kiran. Regarding our jointly published results, I declare the applicant's contribution was prominent. The main articles used in this dissertation have not and will not be used for a Ph.D. dissertation in the future.



28/03/2024 Dr. Laczkó-Dobos Hajnalka

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