氏　　名	陰山　卓哉
学位（専攻分野）	博士（理学）
学 位 記 番 号	総研大甲第1254号
学位授与の日付	平成２１年３月２４日
学位授与の要件	生命科学研究科　基礎生物学専攻 学位規則第６条第１項該当
学 位 論 文 題 目	New Insights Into Selective Autophagy in Yeast: Studies on Lap3
論 文 審 査 委 員	主　査　教授　髙田　慎治 教授　大隅　良典 准教授　林　誠 教授　阪井　康能（京都大学）

論文内容の要旨

「New Insights into Selective Autophagy in Yeast: Studies on Lap3」

Cellular activities require the maintenance of balance between the synthesis and degradation of proteins. Regulation of protein degradation is less understood compared to protein synthesis. The ubiquitin-proteasome system contributes to the selective degradation of short-lived protein. Since most of cellular proteins have long lifetimes, the turnover of long-lived proteins is important to the understanding of cell physiology. Macroautophagy (hereafter simply referred to as autophagy) is an intracellular non-selective degradation system, which is well conserved in eukaryotes; autophagy transports cytoplasmic constituents to the lysosomes/vacuoles for degradation. The autophagic degradation is a cellular response to starvation and plays a role in recycling of cytoplasmic components, which is important for cellular remodeling, development, and differentiation. Screens in the yeast *Saccharomyces cerevisiae* have led to the identification of 31 autophagy-related (*ATG*) genes involved in autophagy. Much progress has been made in the functional analysis of these genes.

Autophagy is initiated by the sequestration of cytoplasmic constituents in a double-membrane structure, termed the autophagosome. Fusion of an autophagosome membrane with the vacuole membrane results in the delivery of an inner vesicle (*i.e.*, autophagic body). Eighteen Atg proteins comprise the core machinery essential for the biogenesis of the autophagosomes. Immuno-electron microscopy has revealed that ribosomes and typical cytosolic marker enzymes are present in the autophagosomes and autophagic bodies at the same densities as in the cytosol, indicating that autophagy is a non-selective and bulk degradation. If degradation of long-lived proteins is exclusively mediated by autophagy, all proteins might be expected to have similar lifetimes. Long-lived proteins, however, have a variety of lifetimes; therefore, the autophagic process would have some selectivity.

Recently, different molecules or complexes are selectively recognized and delivered to the vacuoles via autophagy. Onodera and Ohsumi have reported that Ald6 is preferentially sequestered in autophagosomes and is degraded in vacuoles during prolonged starvation; this mechanism, however, is not understood well. Mechanisms of such cargo selection have been well studied for aminopeptidase I (Ape1), a vacuole-resident enzyme. Ape1 self-assembles and then forms an aggregate-like structure. Ape1 then is selectively incorporated into autophagosomes, and is transported into the vacuole during nitrogen starvation; this process is not degradation but biosynthesis. Thus, little is known about protein that is selectively degraded by yeast autophagy.

To address the issue of selective degradation via autophagy, I focused on leucine aminopeptidase III (Lap3). Lap3 was originally isolated, along with Lap1/Ape2 and Lap4/Ape1, in a genetic screen and is a widely conserved cytoplasmic cysteine protease among eukaryotes. Lap3 self-assembles in the cytosol, and Lap3 also forms homohexameric complex. The first part of this study shows that Lap3 is a selective target of autophagy. When Lap3 tagged with GFP is overexpressed, it forms large aggregates next to the vacuole. Lap3 is transported to the vacuole in a manner dependent on autophagy during nitrogen starvation. Under these conditions, the rate of Lap3 transport is much higher than that of general cytosolic proteins; 27% of [^{35}S]Pho8Δ60, an

indicator of general cytosolic proteins, is transported to the vacuole within 6 h, whereas approximately 50% of the Lap3 is transported to the vacuole in 1.5 h. These results show that Lap3 is selectively transported to the vacuole. I also identified that *ATG11* is involved in Lap3 transport. *ATG11* is essential for selective types of autophagy: selective degradation of peroxisomes (pexophagy) and mitochondria (mitophagy).

Lap3 is apparently reduced after transports to the vacuole at 3 h. I hypothesized that the reduction in Lap3 was a result of degradation in the vacuole. To examine this possibility, the kinetics of Lap3 degradation via autophagic process was measured using temperature-sensitive (*atg1*^{ts}) cell, which shows that most Lap3 is degraded in the vacuole within a couple of hours. Taken together, Lap3 is a novel target of selective degradation mediated by autophagy during nitrogen starvation.

The yeast has a unique system, named the cytoplasm-to-vacuole-targeting (Cvt) pathway. This pathway utilizes common molecular machinery with autophagy under nutrient-rich and fermentable conditions, and constitutively delivers two vacuole-resident enzymes, Ape1 and α-mannosidase (Ams1), to the vacuole via a double-membrane structure (*i.e.*, Cvt vesicle). The latter half of this study shows that the Cvt pathway is involved in not only a biosynthetic process but also the constitutive protein degradation.

The Cvt pathway is enhanced during growth conditions in a medium containing glycerol as non-fermentable carbon source (YPGly). Under the conditions, the amount of vacuole-resident enzymes is increased; the lytic function in the vacuole may be important during YPGly growth, whereas non-selective autophagy is not detectable. I also found that in vacuolar protease-deficient (*pep4*Δ) cells, single-membrane vesicles accumulate in the vacuole under the conditions. The intravacuolar vesicles are detected in *ATG7*-dependent manner, and ribosomes and membranes are apparently excluded from these vesicles.

To investigate the content of the vesicle, I isolated vacuoles from *pep4*Δ cells with or without *ATG7* and subjected to proteomic analysis, leading to the identification of Lap3 and Ape1. When Lap3 tagged with GFP is endogenously expressed, it forms aggregates next to the vacuole during YPGly growth. Lap3 co-localizes with Ape1 and is transported into the vacuole. This transport requires *ATG11* and *ATG19*, which are essential for the Cvt pathway. Atg19, which is a cargo receptor for Ape1, is immunoprecipitated with Lap3; Atg19 is likely to function as a receptor for Lap3 transport. These results show that Lap3 is constitutively transport to the vacuole during vegetative growth and is selectively sequestered in the Cvt vesicles.

I assumed that Lap3 would be unstable in the vacuole, since Lap3 is the target of selective autophagy. To test this assumption, the stability of Lap3 in the vacuole was examined in *atg1*^{ts} cells. The amount of Lap3 in the vacuole decreased to 50% of its initial level within 1.5 h. I also isolated vacuoles from cells with or without *PEP4* and subjected to immunoblot, resulting that Lap3 is not detected in wild-type cell; Lap3 is degraded in the vacuole. Thus, I conclude that the Cvt pathway can function to eliminate certain proteins during vegetative growth.

In this study, I revealed the follow facts; 1) Lap3 is a novel cargo of selective autophagy during nitrogen starvation, and 2) the Cvt pathway plays a role in protein degradation under growth conditions. Recently, protein degradation via autophagic process is thought to play a crucial role in elimination of aberrant protein complex, and this process would be performed

constitutively and selectively. For instance, polyubiquitinated aggregates are recognized by p62, selectively sequestered by autophagosomes and are degraded in lysosomes. In contrast to p62, I do not know yet whether Lap3 are harmful or disadvantageous for yeast cells. As Lap3 is conserved widely in eukaryotes, it may serve as a model protein for analysis of selective autophagy in other organisms as well. Autophagy has traditionally been described as a non-selective degradation process. Several results shown in this thesis is the first study reporting that autophagy can be involved in selective and constitutive protein degradation in yeast cells. This thesis will allow researchers in this field to make new discoveries regarding the regulation and mechanisms of selective and constitutive autophagy.

論文の審査結果の要旨

オートファージーとは、栄養飢餓に応答し、酵母から動植物に至るまで幅広く保存された液胞／リソソーム分解系である。従来、酵母を用いた研究は、グルコース (Glc) など発酵性炭素源を用いた培養条件下で進められてきた。陰山卓哉氏は、まず、出芽酵母を非発酵性炭素源であるグリセロール (Gly) 培地で培養することにより、好気的条件下での液胞機能に関する解析を行った。Gly 培地で培養した細胞では、Glc 培地で培養した細胞に比し、液胞内酵母の発現量が亢進する。また光学顕微鏡観察では液胞内酵素 Pep4p を欠損した株（*pep4*△）の液胞内に動き回る顆粒が観察される。この顆粒は *atg7*D 株では観察されないことからオートファジーに関連した構造体であることが示唆され、Gly 培養時では細胞質から液胞内への輸送経路が作動していることが推測された。次に、電子顕微鏡観察した結果、液胞内に約 200-300nm の構造体が観察された。この構造体に含まれるタンパク質を同定するため *pep4*△から液胞を単離し、質量分析を行った結果、Leucine aminopeptidase III (Lap3) が同定された。Lap3 は真核生物に広く保存された分子である。まず Lap3 の液胞輸送機構を可視化するために、GFP－Lap3 を作製し蛍光顕微鏡観察を行った。GFP－Lap3 は液胞近傍にドットとして観察され、変異体解析から *ATG11*、*ATG19* 依存的に液胞に輸送されることが分かった。さらに生化学的解析からは Atg19 が Lap3p の液胞輸送の受容体として機能し、液胞内に輸送された Lap3 は分解されることがわかった。したがって、Lap3 は選択的にこれまでよく知られている Cvt 経路の標的分子であることが示された。

GAL 代謝関連遺伝子の発現は Gal3 、Gal80 によって制御されるが、Lap3 も負の制御因子として機能することが知られている。Gly 培地ではこれらのタンパク質発現が亢進する。しかし、Gly 培地でのこれらの因子の機能は分かっていない。
Gal3 、Gal80 もまた液胞近傍に集積し、Cvt complex に濃縮され液胞に輸送される。これらの結果、Gal3 および Gal80 も選択的なオートファジーの標的分子であることが示唆された。Lap3 がこれらの因子の細胞内局在及び分解の制御因子として働く可能性を示した。

さらに Glc 培地で培養した細胞では飢餓下に、LapIII がオートファゴソームに選択的に取り込まれ分解されることも明らかにした。この過程にも、Atg11 と Atg19 が重要な役割を担っていることを示した。

本研究により、酵母においても、非発酵性炭素源で増殖するときには、恒常的なオートファジー系として Cvt 経路が分解系として働いていること、グルコース増殖細胞の飢餓誘導性のバルクなオートファジーでも、Lap3 が選択的に取り込まれて分解されることを示した。この時、Lap3 は選択的オートファジーの標的分子であることを明らかにした。今後のオートファジーの選択性に関する研究の端緒を開いた成果として評価され、博士論文として十分であると審査員全員一致で判断した。