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PKA-mediated Autophagy in *Aspergillus fumigates*

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Abstract Autophagy is a ubiquitous, non-selective degradation process in eukaryotic cells that is conserved from yeast to man. Autophagy research has increased significantly in the last ten years, as autophagy has been connected with cancer, neurodegenerative disease and various human developmental processes. Autophagy also appears to play an important role in filamentous fungi, impacting growth, morphology and development. In this review, an autophagy model developed for the *Aspergillus fumigatus* is used as an intellectual framework to discuss autophagy in filamentous fungi. Studies imply that, similar to yeast, fungal autophagy is characterized by the presence of autophagosomes and controlled by the target of rapamycin (Tor) kinase. Autophagy is highly regulated and is under the control of a number of signaling pathways, including the Tor pathway, which coordinates cell growth with nutrient availability. The data shows that autophagy in *A.fumigatus* is also controlled by the cAMP-dependent protein kinase (PKA) pathway. Elevated levels of PKA activity inhibited autophagy and inactivation of the PKA pathway is sufficient to induce a robust autophagy response. In addition, fungal autophagy is apparently involved in protection against cell death and has significant effects on cellular growth and development. However, the only two putative autophagy proteins characterized in filamentous fungi are Atg1 and Atg8. Here we will discuss various strategies used to study and monitor fungal autophagy as well as the possible relationship between autophagy, physiology, and morphological development.

Keywords: *Aspergillus* fumigates; Autophagy; cAMP-dependent protein kinase; Tor kinase

1. Introduction

Filamentous fungi play important roles in health care, agriculture, food production, and bioprocessing. In many of these roles, either by default (in nature) or by design (industrial fermentations), fungi experience nutrient starvation. Such starvation, inturn, can lead to cellular degradation, autolysis, apoptosis and even cell death. However, fungi also possess a nutrient recycling pathway called autophagy that may prolong cellular survival(Klionsky et al., 2016).

Autophagy is a broad term for catabolic processes involving the lysosomal/vacuolar pathway. Autophagy research has become very popular in the last ten years, so much so that it has been referred to as “the new apoptosis”. Connections have even been discovered linking autophagy to cancer, neurodegenerative diseases and human developmental processes(Klionsky, 2011). The term autophagy can describe a number of cellular phenomena including: macroautophagy (non-specific engulfment of cytosolic components by double membrane vesicles which subsequently fuse

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with the vacuole/lysosome where the contents are degraded), microautophagy (direct invagination of cytosolic material into the vacuole/lysosome), pexophagy (degradation of targeted peroxisomes in the vacuole/lysosome), and chaperone-mediated autophagy (degradation of specific cytosolic proteins with assistance from chaperone molecules)(Xia et al., 2015). However, due to a wealth of information from yeast and mammalian cell studies, current literature often uses the term "autophag" to refer to macroautophagy, the non-specific form of this cellular recycling pathway. In addition, the few autophagy studies carried out to date using filamentous fungi have focused exclusively on macroautophagy. Thus in this review, unless otherwise noted, the term autophagy is limited to macroautophagy(Pollack, Harris, & Marten, 2009).

In filamentous fungi, autophagy appears to be involved in nutrient recycling during starvation, and it has been suggested it may also be involved in normal developmental processes. We believe a review of the current autophagy literature, as related to filamentous fungi, may promote interest in considering the role of autophagy in growth and development as well as its relationship to other cellular processes(Akoumianaki et al., 2016). Our intent is to provide an overview of autophagy and the current understanding of its role in filamentous fungi. We follow the path taken by other reviewers of early work on higher eukaryotes, using a simple *A. fumigatus* model for macroautophagy as an initial, intellectual framework to guide our discussion. In the context of this model, we discuss the fungal autophagy proteins that have been characterized to date(Richie & Askew, 2008).

In *A. fumigatus*, autophagy is required for conidiation and hyphal foraging, both of which are adaptive responses to nutrient deficiency that are important to the survival of the organism in its native environment(De Luca et al., 2012). Recent data have also suggested that autophagy contributes tometal ion homeostasis in *A.fumigatus*, but the mechanism by which this is accomplished requires further investigation. Similarly, questions remain about the importance of autophagy to fungal virulence(Chamilos et al., 2016). For example, although autophagy is required for the virulence of some eukaryotic pathogens, it is dispensable for the virulence of others, including *A.fumigatus(Kyrmizi et al., 2013)*. Thus, a comprehensive understanding of how autophagy influences the virulence of some, but not all, eukaryotic pathogens, may provide insight into mechanisms of eukaryotic pathogenesis. Because autophagy in eukaryotic pathogens is an emerging field of investigation, it becomes important to adapt current methodologies to the unique physiology of these organisms. This chapter outlines protocols that have been used to examine autophagy and autophagy-dependent processes in *A. fumigatus(Zhou et al., 2014)*.

2. Overview in autophagy

2.1. Induction and inhibition of autophagy

Autophagy is a broad term covering the processes by which organisms recycle their intracellular components through the vacuole/lysosome, which are essential for cellular survival under conditions that the nutrient supply becomes limiting(Iannitti et al., 2016). The importance of autophagy for mammals is underscored by the notion that this process can be been linked to cancer and neurodegenerative diseases. The term autophagy actually describes multiple cellular phenomena. The best studied of these is nonselective macroautophagy, which involves random uptake of portions of the cytoplasm (cytosol and organelles) into the vacuole/lysosome for recycling (de Luca et al., 2014). In many eukaryotes, macroautophagy is not only a survival mechanism, but the recycled building blocks are also utilized during differentiation, for example, in spore formation in some yeast species. Macroautophagy is defined by the formation of a double membrane structure that engulfs a portion of cytoplasm, resulting in the formation of a vesicular structure termed an autophagosome(Sprenkeler, Gresnigt, & van de Veerdonk, 2016). Subsequently, the outer membrane layer of the autophagosome will fuse

with the vacuolar membrane and a single membrane structure, commonly referred to as an autophagic body, enters the lumen of the vacuole. There, this structure is lysed and its contents recycled by vacuolar hydrolases to replenish the nutrient depletion or to stimulate cellular differentiation(Chamilos et al., 2016). Nonselective incorporation of cytoplasmic components by the vacuole can also take place via microautophagy, a process that is defined by the direct uptake of a portion of the cytoplasm by vacuolar invagination(Martinez et al., 2015).

As in other organisms, fungal autophagy is typically induced by nutrient (e.g., carbon, nitrogen) starvation. It can also be induced by treatment with the macrolide rapamycin, with concentrations between 200 and 500 ng/ml often used in fungal culture. However, we note that these concentrations have typically been used in static or solid cultures whereas significantly higher rapamycin concentrations (>60 ug/ml) may be necessary to inhibit growth in liquid culture, particularly for several species of *Aspergillus(Oikonomou et al., 2016).*

Autophagy can be inhibited either chemically or by the disruption of genes coding for autophagy proteins. In the fungus *Podospora anserina*, disruption of genes encoding Atg1 and Atg8 homologs resulted in similar phenotypic changes in growth and differentiation. This is consistent with findings from *Aspergillus fumigates* where disruption of different autophagy genes resulted in similar changes in phenotypic traits, inhibition of filamentous growth. Autophagy may also be inhibited chemically(X. Li et al., 2016). The class I and class III inhibitors of phosphotidylinositol 3-kinases, 3-methyladenine (3-MA) and wortmannin, respectively, have been used to inhibit autophagy in mammalian and plant cells. Yet some fungal species are apparently resistant to wortmannin, as this metabolite does not appear to inhibit autophagy in *Aspergillus* or *Neurospora* species(Zelante et al., 2012).

2.2. Autophagy in cellular degradation

In general, autophagy serves as a major eukaryotic process for protein degradation, and is the only pathway for degradation of long-lived proteins and whole organelles, particularly those that are damaged or obsolete. In filamentous fungi, autophagy is typically accompanied by vacuolar enlargement(Palmer, Askew, & Williamson, 2008). Vacuoles are degradative organelles, and hyphal vacuolation has been shown to increase rapidly in nutrient-starved *A.fumigatus* mycelia. Autophagic bodies are found inside vacuoles when autophagy is induced, but not in mutant strains defective of autophagy, such as in the *A. fumigates* DAfatg1 strain. However, vacuolation is not suppressed in *P. anserina* DPa*atg1* nor DPa*atg8* strains, which suggests that vacuolation itself is not dependent upon autophagy(Tam et al., 2016).

2.3. Autophagy in cell death

While autophagy has been called type II programmed cell death (PCD) or autophagic cell death, there is evidence that autophagy may play both causative and preventative roles in cell death(Yan et al., 2013). For example, in the plant pathogen *Magnaporthe grisea*, autophagy is required for spore collapse (cell death) during host infection. In contrast, autophagy does not appear to be a requirement for cell death in *P. anserina*, although it is induced during cell death by incompatibility (a model for type II PCD). In fact, autophagy may have a pro-survival function by eliminating “pro-death” signals that move from damaged organelles or compartments to adjacent healthy cells(Richie et al., 2007).

Autophagy also appeared to be connected to autolysis, a naturalself-degradation process by endogenous hydrolase activity, whose prolonged leads to cell death. Many factors affect fungal autolysis in *A.fumigatus*, such as aging, PCD, hyphal differentiation, nutrient limitation, and physical stress; however, the molecular mechanisms of autolysis have not yet been elucidated. Autophagy has been shown to precede autolysis, and may possibly play a role in the early onset of

autolysis(Fuller, Zhao, Askew, & Rhodes, 2009).

2.4. Autophagy in cellular differentiation

In a number of species, autophagy is apparently involved in determining cell architecture during differentiation and development. Similarly in filamentous fungi, defects in autophagy genes influence morphogenesis and morphology. In *A. fumigatus*, mutants with deletions of Atg1 or Atg8 genes consistently show reduced numbers of aerial hyphae, disrupted conidiation, and delayed germination; such effects also have been shown in *M. grisea*, *P. anserine*, and *A. oryzae*. And deletion of autophagy genes also inhibits formation of sexual reproductive organs(Dice, 2010). These findings strongly suggest that in several species of filamentous fungi a functional autophagy pathway is required for correct cellular differentiation to occur. Autophagy also appears to play a role in differentiation of fungal infectous structures. In *A.fumigatus*, a deletion mutant for clk1 (a homolog of Atg1) shows defects in leaf cuticle penetration. However, autophagy does not appear to always be involved in cellular differentiation. For example, in the filamentous yeast *Candida albicans*, autophagy disruption, in an Datg9 mutant does not affect hyphal differentiation or formation of chlamydospores(Bestebroer, V'Kovski, Mauthe, & Reggiori, 2013). Thus, the role of autophagy in fungal differentiation may, to some degree, be species dependant.

3. Regulation between autophagy and PKA

Autophagy was initially identified as a cellular response to nutrient deprivation and is essential for cell survival during these periods of starvation. Autophagy is highly regulated and is under the control of a number of signaling pathways, including the Tor pathway, that coordinate cell growth with nutrient availability. These pathways appear to target a complex of proteins that contains the Atg1 protein kinase. The studied have showed that autophagy in *A.fumigatus* is also controlled by the cAMP-dependent protein kinase (PKA) pathway(Politi et al., 2014). Elevated levels of PKA activity inhibited autophagy and inactivation of the PKA pathway was sufficient to induce a robust autophagy response. The data showed that in addition to Atg1, PKA directly phosphorylates Atg13, a conserved regulator of Atg1 kinase activity. This phosphorylation regulates Atg13 localization to the preautophagosomal structure, the nucleation site from which autophagy pathway transport intermediates are formed. Atg13 is also phosphorylated in a Tor-dependent manner, but these modifications appear to occur at positions distinct from the PKA phosphorylation sites identified here. In all, the studies indicated that the PKA and Tor pathways function independently to control autophagy in *A.fumigatus(Stephan, Yeh, Ramachandran, Deminoff, & Herman, 2009)*.

3.1. Model for *A.fumigatus* autophagy

As the molecular mechanisms of autophagy continue to be elucidated(currently, more than 30 autophagy genes have been identified), increasingly complex models have been proposed. However, as only a limited number of autophagy studies have been carried out in filamentous fungi, we put forward a simple autophagy model that consists of four sequential steps (1) induction of autophagy through Tor kinase, (2) formation of autophagosomes and sequestration of cytoplasm and organelles, (3) docking of autophagosome and fusion with vacuole, and (4) breakdown of autophagic bodies in the vacuole into macromolecules ready for recycling(Geng & Klionsky, 2010).

3.2. Tor pathway

Tor is involved in nutrient sensing and regulation of transcription, translation, and protein degradation. It is part of a signal transduction pathway conserved from *A.fumigatus* to humans. Tor can be inhibited by the immunosuppressive macrolide rapamycin by forming a ternary complex between Tor, rapamycin, and the peptidyl-prolyl isomerase FKBP12(Calvo-Garrido, Carilla-Latorre, Mesquita, & Escalante, 2011). Tor inhibition by rapamycin results in upregulation of

autophagy-related genes (homologs of the Podospora idi7/Atg8), but also upregulation of genes involved in transcription, secondary metabolism, and stress response. Tor inhibition by rapamycin also results in the downregulation of genes involved in ribosome biogenesis and translation initiation. Tor also appears to be essential for viability, as disruption of the Tor gene is lethal in *A.fumigatus(Biazik, Yla-Anttila, Vihinen, Jokitalo, & Eskelinen, 2015)*.

3.3. Inactivation of the PKA Pathway Is Sufficient to Induce Autophagy**.**

Elevated levels of PKA activity have been shown to inhibit the autophagy process. The studies tested whether the inactivation of this signaling pathway was also sufficient to induce autophagy(Turturici, Tinnirello, Sconzo, & Geraci, 2014). To shutdown PKA signaling, the study used an inducible form of a dominant negative allele of *RAS2*, known as $RAS2^{ala22}$. In *A.fumigatus*, the Ras proteins, Ras1 and Ras2, regulate cAMP production, and thus PKA activity, by directly stimulating adenylyl cyclase. The $RAS2^{ala22}$ allele used here was under the control of the promoter from the *MET3* gene, a locus that is repressed when methionine is in the growth medium. The study found that autophagy was efficiently induced upon expression of the $RAS2^{ala22}$ protein and that the kinetics of induction were similar to that observed with rapamycin treatment(Gao et al., 2010). Moreover, this $RAS2^{ala22}$-mediated induction was dependent upon the presence of Atg1. To directly compare the effects of inhibiting the Tor and PKA pathways, the study used a concentration of rapamycin that produced a growth arrest similar to that observed in the *MET3-*$RAS2^{ala22}$ strain. Therefore, a decrease in PKA function that was sufficient to arrest *A.fumigatus* growth resulted in a concomitant increase in autophagy activity. These results are consistent with a previous study that used a galactose-inducible formof this $RAS2^{ala22}$ allele(Subramani & Malhotra, 2013).

3.4. The Pka and Tor pathways independently target the Atg13 protein

The simultaneous inactivation of both the PKA and Tor pathways produced a more rapid and greater induction of autophagy than was observed with the loss of either pathway alone. This result is consistent with these pathways working independently of each other to control autophagy. To examine this possibility, the studies tested how shutting down one of these pathways would influence the activity of the other(Duke et al., 2014). They used the PKA- and Tor-dependent phosphorylations of Atg13 as reporters for the activity of the respective signaling pathways. The research found that the in vivo level of Atg13 phosphorylation by PKA was not diminished upon rapamycin treatment. In addition, the inactivation of the Ras/PKA pathway did not result in a loss of the Tor-dependent phosphorylation present on Atg13. This latter phosphorylation causes Atg13 to run as a broad smear on SDS-polyacrylamidegels. This smear rapidly collapses into a tight, faster-migratingb and upon rapamycin treatment. Finally, the in vitro phosphorylation of Atg13 by PKA did not alter the mobility of this protein in SDS-polyacrylamide gels. Therefore, although the precise locations of the Tor-dependent phosphorylation sites on Atg13 have yet to be identified, these positions appear to be distinct from the PKA sites described here(M. Li et al., 2013). In all, these data are consistent with the Tor and PKA pathways working independently to control autophagy.

4. Future perspectives

Autophagy in *A.fumigatus* is still in its infancy. Although many autophagy proteins identified in other species share homology in filamentous fungi, their function may not be well conserved. Therefore, in order to better understand the mechanistic details involved in fungal autophagy, it is important first to identify the necessary and assignable proteins and then to investigate the functional role of these proteins.

Future Autophagy studies of filamentous fungi will need to address the following questions: How do

autophagy and proteins affect cell morphology and physiology? Can autophagy affect cell growth, branching, and cell wall properties? What is the relationship between autophagy and other stress responses in filamentous fungi? By answering these questions, you can better understand autophagy in filamentous fungi.

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