Full Paper

Carbon and nitrogen depletion-induced nucleophagy and selective autophagic sequestration of a whole nucleus in multinucleate cells of the filamentous fungus *Aspergillus oryzae*

(Received August 13, 2016; Accepted September 1, 2016; J-STAGE Advance publication date: March 22, 2017)

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Introduction

Autophagy is a conserved cellular degradation process in eukaryotes, in which cytoplasmic components and organelles are digested in vacuoles/lysosomes. Recently, autophagic degradation of nuclear materials, termed “nucleophagy”, has been reported. In the multinucleate filamentous fungus *Aspergillus oryzae*, a whole nucleus is degraded by nucleophagy after prolonged culture. While developing an H2B-EGFP processing assay for the evaluation of nucleophagy in *A. oryzae*, we found that nucleophagy is efficiently induced by carbon or nitrogen depletion. Microscopic observations in a carbon depletion condition clearly demonstrated that autophagosomes selectively sequester a particular nucleus, despite the presence of multiple nuclei in the same cell. Furthermore, AoNsp1, the *A. oryzae* homolog of the yeast nucleoporin Nsp1p, mainly localized at the nuclear periphery, but its localization was restricted to the opposite side of the autophagosome being formed around a nucleus. In contrast, the perinuclear ER visualized with the calnexin AoClxA was not morphologically affected by nucleophagy. The findings of nucleophagy-inducing conditions enabled us to characterize the morphological process of autophagic degradation of a whole nucleus in multinucleate cells.

Key Words: *Aspergillus oryzae*; autophagy; filamentous fungi; multinucleate cell; nucleophagy

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In mammals, the mutant alleles of the genes encoding lamin A and emerin, leading to muscular dystrophy, cause the formation of large perinuclear autophagosomes engulfing nuclear components (Park et al., 2009). Nuclear membrane blebs containing lamin B1, induced by oncogenic stress, is also degraded by autophagy (Dou et al., 2015). In addition, it has been reported that autophagy is involved in the elimination of micronuclei (Rello-Varona et al., 2012), which are formed by mitotic abnormalities when exposed to genotoxic agents (Erenpreisa et al., 2012). In these cases, nucleophagy involves the degradation of abnormal nuclear structures, which are sequestered into the autophagosome in a manner termed macroautophagy (Mijaljica and Devenish, 2013).

In the budding yeast *Saccharomyces cerevisiae*, nucleophagy is induced by nitrogen starvation (Mijaljica
Aspergillus oryzae strains used in this study

| Strain name | Genotype | Reference |
|-------------|----------|-----------|
| RIB40       | Wild strain | Machida et al. (2005) |
| NSR29/1-1   | muidD::sC   | Yamada et al. (1997) |
| DASH4GCN    | muiD::sC   | Kikuma et al. (2006) |
| HREA        | muiD::sC   | This study |
| NcG94/8     | muiD::sC   | This study |
| NcG94/8Cha  | muiD::sC   | This study |
| CRG84        | muiD::sC   | This study |

Table 1. Aspergillus oryzae strains used in this study.

for the detection of nucleophagy in A. oryzae, and determined the nucleophagy-inducing conditions. Furthermore, we inves-

and Devenish, 2013; Mochida et al., 2015). Three modes of typical nucleophagy have been reported: piecemeal microautophagy of the nucleus (PMN) (Roberts et al., 2003), late nucleophagy (LN) (Mijaljica et al., 2012), and Atg39-dependent nucleophagy (Mochida et al., 2015). Short-term nitrogen starvation (~3 h) induces PMN, in which small blebs of the nucleus are directly engulfed into the vacuoles by a microautophagic process (Roberts et al., 2003). The initiation of PMN occurs at the nucleus-vacuole (NV) junctions (Krick et al., 2008; Roberts et al., 2003). LN degrades the nucleoplasm through a microautophagic process in the NV junction-independent manner, and is detected only after the prolonged culture of nitrogen starvation (20–24 h) (Mijaljica et al., 2012). Atg39-dependent nucleophagy has been described as the selective degradation of the perinuclear endoplasmic reticulum (ER)/nuclear envelope together with part of the nucleoplasm, by macroautophagy (Mochida et al., 2015).

Nucleophagy of a whole nucleus was first found in the prolonged cultured (48–72 h) cells of Aspergillus oryzae, the industrial filamentous fungus used in Japanese traditional fermentation (Shoji et al., 2010). Additionally, it was reported that nucleophagy of a whole nucleus occurs during appressorium formation required for plant infection in the rice blast fungus Magnaporthe oryzae (He et al., 2012) and after vegetative cell fusion and during nitrogen starvation in another plant pathogen Fusarium oxysporum (Corral-Ramos et al., 2015). In A. oryzae, the morphological process of nucleophagy has been characterized by fluorescence microscopy under the expression of an enhanced green fluorescence protein (EGFP)-fused AoAtg8, an orthologue of the autophagosomal marker S. cerevisiae Atg8, (EGFP-AoAtg8) and the nuclear marker mDsRed-fused histone H2B (H2B-mDsRed) (Shoji et al., 2010); AoAtg8-positive crescent- and cup-shaped autophagosome precursors are formed in the vicinity of the mDsRed-labeled nucleus, and then a ring-like autophagosome engulfs the whole nucleus. Subsequently, the nucleus sequestered by the autophagosome is delivered into the vacuole, followed by the dispersal of the nuclear materials throughout the vacuolar lumen. The nucleophagy of a whole nucleus has not been reported in mammals and yeasts, since such cells are typically mononuclear. It is thought that the degradation of one nucleus does not cause lethality in the multinucleate cells of filamentous fungi. However, it was difficult to efficiently observe how nucleophagy selectively degrades nuclei in A. oryzae, since the nucleophagic process is not synchronized in individual hyphae during the prolonged culture. In addition, although part of the nuclear membrane is degraded by nucleophagy in mammals and yeast, it is unclear how the perinuclear structures are processed during the degradation of a whole nucleus in filamentous fungi. For investigating the morphological characteristics of nucleophagy in the multinucleate cell, it is important to identify the culture conditions that efficiently induce nucleophagy.

In the present study, we developed an EGFP-fused histone H2B (H2B-EGFP) processing assay for the detection of nucleophagy in A. oryzae, and determined the nucleophagy-inducing conditions.
tigated the morphological process of perinuclear structures during nucleophagy.

Materials and Methods

Aspergillus oryzae strains and growth media. The A. oryzae strains used in this study are listed in Table 1. The A. oryzae wild strain RIB40 (Machida et al., 2005) was used as a DNA donor. The strain HREA, co-expressing the fusion proteins H2B-mDsRed and EGFP-AoAtg8 (Shoji et al., 2010), was used for microscopic observations. The Czapek-Dox (CD) medium [0.3% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, and 2% glucose (pH 5.5)] was used as the selective medium for transformation and for fluorescence microscopy. A DYP medium [(2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O (pH 5.5)] and CD media lacking NaN₃ (CD-N) or glucose (CD-C) were used for the H2B-mDsRed processing assay and for fluorescence microscopy.

H2B-EGFP processing assay. For the construction of strains expressing H2B-EGFP, the plasmid pBAsCH2B containing the h2b-egfp fusion gene (Maruyama et al., 2001) and pNR10 containing the niaD marker gene (Escaño et al., 2009) were coin-introduced into the A. oryzae strain NS4 (Yamada et al., 1997), generating the strain HGsCN. The Aoatg8 deletion strain ΔAoatg8-1-1 (Kikuma et al., 2006) was transformed with the same two plasmids pBAsCH2B and pNR10, generating the strain DA8HGsCN. Approximately 2.5 × 10⁴ conidia of these strains were inoculated in 100 ml of DYP medium and cultured at 30°C for 24 h. The medium was then replaced with either 100 ml of CD, CD-N, or CD-C media, and the mycelia were further incubated at 30°C for 2, 4 and 6 h. Subsequently, 0.25 g wet weight of mycelia were frozen in liquid nitrogen, and then thoroughly ground into powder using a Multi-beads shocker (Yasui Kikai, Osaka, Japan). The mycelial powder was suspended in 1 ml of extraction buffer [50 mM Tris-HCl (pH 7.5), phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 200 × g at 4°C for 10 min. Then, the supernatant (mycelial protein extracts) was subjected to Western blotting using the anti-GFP mouse monoclonal antibody (1:5,000 dilution; Clontech, Mountain View, CA, USA) and peroxidase-labeled mouse anti-immunoglobulin G antibody (1:500 dilution; Vector Laboratories, Burlingame, CA, USA) as primary and secondary antibodies, respectively.

Construction of strains for microscopic analysis of perinuclear structures. To examine the localization of AoNsp1, a plasmid pgPaNsplG for the expression of the Aonspl-egfp fusion gene under the control of the amyB promoter was constructed using the MultiSite Gateway cloning system (Invitrogen, Carlsbad, CA, USA). The putative coding region of Aonspl was then amplified by PCR using the primer pair Aonspl-F (5′-GGGGACAAAGTTTTGATACAAAAAGCAAGGCTATGTCTTTTTCATTCGGTGCTCCA-3′) and Aonspl-R (5′-GGGGACACCTTTTGATACAAAAAGCAAGGCTATGTCTTTTTCATTCGGTGCTCCA-3′). The amplified fragment was cloned into the entry vector pDONR_221 (Invitrogen) by the Gateway BP clonase reaction, generating the center entry clone plasmid pgENsp1. The 5′ and 3′ entry clones containing the amyB promoter (pg5′Pa; Mabashi et al., 2006) and egfp (pgEE; Mabashi et al., 2006), respectively, were mixed together with pgENsp1 and the destination vector pgDN containing the niaD marker gene (Mabashi et al., 2006) for the Gateway LR clonase reaction. The resulting plasmid pgPaNsplG was then introduced into the A. oryzae strain NSRKu70-1-1A (Escaño et al., 2009). One transformant was designated NspG.

To simultaneously visualize the nuclei with the AoNspl localization, the plasmids pgH2BR containing the h2b-mdsred fusion gene and the A oryzae sC marker (Shoji et al., 2010) was introduced into the strain NspG. In the generated strain NspGHR, nuclei were labeled with mDsRed.

To simultaneously visualize the ER or autophagosomal structures with the AoNspl localization, the plasmids pgClxAdsred containing the AoclxA-mdsred fusion gene (Kimura et al., 2011), or pgPaRA8 containing the mdsred-Aoatg8 fusion gene (Tadokoro et al., 2015), were introduced into the strain NspG. In the generated strains NspGHR and NspGRA8, ER and autophagosomal structures were labeled with mDsRed, respectively.

To simultaneously visualize ER and autophagosomes, the plasmids pgClxAdsred and pgEGA8 containing the egfp-Aoatg8 fusion gene were co-introduced into the strain NSRKu70-1-1A. One positive transformant was designated CIRGA8.

The strains NspGHR, CIRNsplG, NspGRA8, and CIRGA8 were observed by fluorescence microscopy as mentioned below.

Fluorescence microscopy and image analysis. For microscopic analysis, approximately 1.0 × 10⁴ conidia were inoculated in 100 μl of CD medium in a glass-based dish (Asahi Techno Glass, Chiba, Japan), which were then incubated at 30°C for 24 h. Glucose was used as the carbon source in the medium to have the moderately expressing level of EGFP- or mDsRed-fusion proteins under the control of the amyB promoter (Tada et al., 1991). Subsequently, the CD medium was replaced with fresh CD, CD-N, or CD-C media, and the mycelia were further incubated at 30°C for 30 h. Fluorescence microscopic analysis was performed using an IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with 100 × Neofluor objective lenses (1.30 numerical aperture), 488 nm (Furukawa Electric, Tokyo, Japan) and 561 nm (Melles Griot, Rochester, NY, USA) semiconductor lasers, GFP, DsRed, and DualView filters (Nippon Roper, Tokyo, Japan), a CSU22 confocal scanning system (Yokogawa Electronics, Tokyo, Japan), and an Andor iXon cooled digital CCD camera (Andor Technology PLC, Belfast, Ireland). Images were analyzed with Andor iQ software (Andor Technology PLC).

Results and Discussion

Development of the H2B-EGFP processing assay for monitoring nucleophagy in A. oryzae

In S. cerevisiae, GFP-fused Pex14 (Pex14-GFP) and
Om45 (Om45-GFP) processing assays are used to monitor pexophagy and mitophagy, respectively (Cheong and Klionsky, 2008; Kanki et al., 2009), in which the degradation of these organelles is evaluated by the detection of free GFP derived from the fusion proteins. Due to the relative resistance to degradation of GFP by vacuolar hydrolases, free GFP moieties are accumulated in the vacuole, which can be detected by Western blotting. To determine the culture conditions that induce nucleophagy in A. oryzae, we sought to develop the processing assay to evaluate nucleophagy by using the fusion protein H2B-EGFP, which has been used as a nuclear marker in A. oryzae (Maruyama et al., 2001; Shoji et al., 2010).

As the prolonged culture induces nucleophagy in A. oryzae, we were interested in developing a processing assay to evaluate nucleophagy by using the fusion protein H2B-EGFP, which has been used as a nuclear marker in A. oryzae (Maruyama et al., 2001; Shoji et al., 2010).

To confirm whether the processing was dependent on autophagy, we examined the processing of H2B-EGFP in the ΔAoatg8 deletion strain, in which autophagy including nucleophagy is defective (Shoji et al., 2010). As shown in Fig. 1B, no free EGFP was detected in the ΔAoatg8 strain even after 6 h of carbon or nitrogen depletion. This result indicates that the processing of H2B-EGFP is dependent on autophagy, and this assay reflects the progression of nucleophagic flux.

**Nucleophagy is rapidly induced by carbon depletion in A. oryzae**

To confirm the induction of nucleophagy by carbon or nitrogen depletion, we observed hyphae of the strain co-expressing H2B-mDsRed and EGFP-AoAtg8 (Shoji et al., 2010) by fluorescence microscopy (Fig. 2). After 24 h of cultivation in a CD medium, the medium was replaced with fresh CD, CD-C, or CD-N media, and hyphae of the strain were further incubated for 2, 4 and 6 h. In the fresh CD medium, EGFP fluorescence derived from EGFP-AoAtg8 was detected in vacuoles after 4 h of further incubation, while the fluorescence was already found in vacuoles after 2 h in CD-C and CD-N media (Fig. 2). Based on the previous observation of EGFP-AoAtg8 as an autophagic indicator (Kikuma et al., 2006), the results reveal that autophagy is induced earlier in carbon or nitro-
Nucleophagy induction in \textit{A. oryzae}.

Next, mDsRed fluorescence derived from H2B-mDsRed was observed, since its accumulation in the vacuole is indicative of nucleophagy (Shoji et al., 2010). mDsRed fluorescence was not detected in vacuoles within 6 h of incubation in the fresh CD medium (Fig. 2; CD). In CD-C and CD-N media, the mDsRed fluorescence was detected in vacuoles after 2 and 6 h, respectively (Fig. 2; CD-C and CD-N). These observations demonstrate that nucleophagy is induced in carbon or nitrogen depletion, and that carbon depletion is an efficiently inducing condition for nucleophagy. This is consistent with the data from the H2B-EGFP processing assay (Fig. 1), and therefore the validity of the assay was confirmed for evaluating nucleophagy in \textit{A. oryzae}.

Hyphae of most filamentous fungi, including \textit{A. oryzae}, are divided into cells by septa, and the cells contain multiple nuclei. However, it was difficult to observe how multiple nuclei were degraded by nucleophagy in the cells of old hyphae due to the decreased number of nuclei during the prolonged culture. We assumed that the induction of nucleophagy by carbon depletion allows us to observe the nucleophagic process in the multinucleate cell. Thus, we observed the sequestration of nuclei by autophagosomes after 1.5 h of carbon depletion. Autophagosomes and nuclei were labeled with the fusion proteins EGFP-AoAtg8 and H2B-mDsRed, respectively. The crescent- and cup-shaped autophagosome precursors (Figs. 3A and B) and ring-like autophagosomes (Fig. 3C) were formed to sequester one nucleus in the multinucleate cell. In addition, only one vacuole accumulated the mDsRed fluorescence derived from H2B-mDsRed, but...
other vacuoles in the same cell did not, suggesting that at least one nucleus was delivered into the particular vacuole and degraded (Fig. 3D). These observations demonstrate that one nucleus is selectively sequestered for nucleophagy in the multinucleate cell.

**Morphological characteristics of perinuclear structures during nucleophagy**

In mammals and yeast, part of the perinuclear structures, such as the perinuclear ER/nuclear envelope, is degraded by nucleophagy (Mijaljica and Devenish, 2013), and hence we investigated how perinuclear structures are processed during the nucleophagy of a whole nucleus in *A. oryzae*. First, nuclear pores were visualized by expressing the EGFP-fused orthologue of *S. cerevisiae* Nsp1p, an FG-repeat nucleoporin, which localizes at the nuclear pores...
Nucleophagy induction in *A. oryzae* (Nehrbass et al., 1990). The Nsp1p orthologue in *A. nidulans* (An-Nps1) localizes to the nuclear periphery (De Souza et al., 2004). Therefore, we decided to use *A. oryzae* Nsp1 as a marker to visualize the nuclear pores. A gene homologous to *S. cerevisiae* NSP1, AO090120000255 (named as Aonsp1), was identified from the *A. oryzae* genome database (http://www.bio.nite.go.jp/dogan/project/view/AO). Aonsp1 encodes a polypeptide of 823 amino acids, which shares a 23% identity with *S. cerevisiae* Nsp1p.

When nuclei were simultaneously visualized by H2B-mDsRed, the fluorescence of AoNsp1-EGFP was found at the nuclear periphery (Fig. 4A), which is similar to the localization pattern of *A. nidulans* An-Nps1 (De Souza et al., 2004). In addition, the Nsp1-EGFP fluorescence was detected as discontinuous ring-like structures, which partially co-localized with the ring-like perinuclear ER labeled with mDsRed-fused AoClxA, the ER marker calnexin (Kimura et al., 2010) (Fig. 4B). These observations reveal that the fusion protein AoNsp1-EGFP localizes at the nuclear periphery probably targeting to the nuclear pores.

We observed nucleophagy in the strain co-expressing AoNsp1-EGFP and mDsRed-AoAtg8. After culturing the strain for 24 h at 30°C in a CD medium, the hyphae were shifted into CD-C and further cultured for 1.5 h to induce nucleophagy. The localization of AoNsp1-EGFP at the nuclear periphery became restricted to the opposite side of the crescent- and cup-shaped autophagosome precursors labeled with mDsRed-AoAtg8 (Fig. 4C; upper and middle panels). When the formation of the ring-like autophagosome was completed, a few of the dots labeled with AoNsp1-EGFP remained along the autophagosome (Fig. 4; lower panels).

Next, the morphology of the perinuclear ER during nucleophagy was observed with the strain co-expressing the fusion proteins AoClxA-mDsRed and EGFP-AoAtg8. The ring-like morphology of the perinuclear ER labeled with AoClxA-mDsRed exhibited no apparent difference even when encircled by the cup-shaped and ring-like autophagosomes of EGFP-AoAtg8 (Fig. 5). These results indicate that the localization of the nuclear pore protein, but not the morphology of the perinuclear ER, is affected during the formation of the autophagosome around a nucleus.

In the present study, we have developed a method for the evaluation of nucleophagy in populations of mycelia, which was termed the H2B-EGFP processing assay, and we have demonstrated for the first time that carbon depletion, rather than nitrogen depletion, efficiently induces nucleophagy in *A. oryzae*. This finding enabled us to show that one nucleus is selectively sequestered into the autophagosome for nucleophagy in the multinucleate cell. The identification of receptors for recognition of nuclei would lead to uncovering how one particular nucleus is selected from multiple nuclei for nucleophagy.

In addition, we investigated the morphological characteristics of perinuclear structures during nucleophagy; the localizations of the nuclear pore protein and perinuclear...
ER are differently affected during the formation of nucleophagic autophagosomes. The observations raise the possibilities that the elongation of nucleophagic precursors excludes the nuclear pore protein, or that the formation of nucleophagic autophagosomes occurs at a region devoid of the protein. This issue could be resolved by a detailed investigation of autophagy-deficient mutants. Moreover, high-resolution analysis by electron microscopy would help in achieving a detailed understanding of how perinuclear structures are processed by nucleophagy. Further molecular biological and morphological analyses are expected to provide new insights into the mechanism and physiological roles of nucleophagy in filamentous fungi.

Acknowledgments

This work was supported by the Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan under Grant No. 25292045.

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