Running title: TORC1 responds to proteotoxic stress

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TORC1 regulates autophagy induction in response to proteotoxic stress in yeast and human cells

Author names:

Kazuki Suda[1], Atsuki Kaneko[2], Mitsugu Shimobayashi[3], Akio Nakashima[4], Tatsuya Maeda[5], Michael N. Hall[3] and Takashi Ushimaru[1,2,*]

Affiliations:

[1]*Faculty of Science, Department of Biological Science, Shizuoka University, Shizuoka, 422-8021, Japan*

[2]*Course of Biological Science, Department of Science, Graduate School of Integrated Science and Technology, Shizuoka University, Shizuoka, 422-8021, Japan*

[3]*Biozentrum, University of Basel, 4056 Basel, Switzerland, Switzerland*

[4]*Biosignal Research Center, Kobe University, Kobe 657-8501, Japan*

[5]*Department of Biology, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-3192*

*Correspondence: ushimaru.takashi@shizuoka.ac.jp

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Abbreviations: AZC, azetidine-2-carboxylic acid; CDK, cyclin-dependent kinase; GFP, green fluorescent protein; mTOR, mammalian TOR; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; PAS, pre-autophagosomal structure; PQC, protein quality control; TOR, target of rapamycin; TORC1, target of rapamycin complex 1; TORC2, target of rapamycin complex 2.

Abstract

Misfolded and aggregated proteins are eliminated to maintain protein homeostasis. Autophagy contributes to removal of protein aggregates. However, if/how proteotoxic stress induces autophagy is poorly understood. Here we show that proteotoxic stress after treatment of azetidine-2-carboxylic acid (AZC), a toxic proline analog, induces autophagy in budding yeast. AZC treatment attenuated target of rapamycin complex 1 (TORC1) activity and thereby Atg13, a key factor of autophagy, was dephosphorylated. By contrast, AZC treatment did not affect target of rapamycin complex 2 (TORC2). Proteotoxic stress also induced TORC1 inactivation and autophagy in fission yeast and human cells. This study suggests that TORC1 is a conserved key factor to cope with proteotoxic stress in eukaryotic cells.

Introduction

Maintenance of protein homeostasis (proteostasis) under normal and adverse stress (e.g., high temperature) conditions is essential for all living organisms [1, 2]. To preserve proteostasis, misfolded or denatured proteins are refolded or degraded by the protein quality control (PQC) system, consisting of molecular chaperones, the ubiquitin proteasome system, and autophagy [2, 3]. Severe proteotoxic stress or dysfunction of the PQC system causes accumulation of cellular misfolded proteins and protein aggregates, causing impairments of cellular functions and cell death. Many diseases, including neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease, are connected with accumulation of toxic misfolded proteins [4, 5].

Target of rapamycin complex 1 (TORC1), a nutrient-activating protein kinase, promotes diverse cellular metabolic events for cell growth, including ribosome biogenesis and translation [6, 7]. TORC1 activity is attenuated in various stresses, such as nutrient starvation, DNA damage, and hypoxia [8, 9]. In addition, it has been reported that proteotoxic stress after azetidine 2-carboxylic acid (AZC) attenuated mammalian TORC1 (mTORC1) activity in human cells [10, 11]. AZC is a toxic proline analog that is incorporated into synthesized protein competitively and can generates misfolded proteins and protein aggregates [12, 13]. AZC treatment is ideal for analysis of bulk proteotoxic stress response as compared with heat shock treatment, since heat shock

impacts on not only proteins but also other various cellular components. For instance, mRNA forms stress granules and P-bodies upon heat shock [14, 15].

Proteotoixc stress-induced TORC1 attenuation might be important for alleviation of proteotoxic stress, since TORC1 attenuation causes not only reduction in protein synthesis but also induction of autophagy [16, 17]. In the budding yeast, *Saccharomyces cerevisiae*, TORC1 phosphorylates Atg13 in nutrient-rich conditions, repressing autophagy induction [18, 19]. When nutrient is depleted, TORC1 is inactivated and Atg13 is promptly dephosphorylated by PP2A and Cdc14 protein phosphatases [20, 21], which in turn promotes formation of the Atg1 kinase complex (containing Atg1 and Atg13) triggering autophagy induction. Thus, the TORC1–Atg13–Atg1 axis is a critical pathway for starvation-induced autophagy. A similar signaling pathway is conserved in human cells. However, it is unknown whether AZC-mediated attenuation of TORC1 activity is conserved among eukaryotes. In addition, whether and how proteotoxic stress induces autophagy is unclear. Here we show that AZC treatment moderately attenuates TORC1 activity and induces autophagy in budding yeast. By contrast, AZC treatment did not affect target of rapamycin complex 2 (TORC2), a different target of rapamycin (TOR) complex. AZC treatment also induced TORC1 inactivation and autophagy in fission yeast and human cells. This study suggests that TORC1 is a key factor to cope with proteotoxic stress in eukaryotic cells.

Results

Proteotoxic stress induces autophagy in budding yeast

First, we examined whether autophagy is induced in response to proteotoxic stress after AZC treatment in budding yeast cells. Rapamycin treatment that strongly induces autophagy (monitored by green fluorescent protein [GFP]-Atg8 processing) [22] was used as the control. Free GFP was remarkably produced from GFP-Atg8 after rapamycin treatment (Fig. 1A), as described [22]. After treatment with 2.5 mM or 5 mM AZC that evoked effectively proteotoxic stress in yeast cells [23], free GFP was slightly produced. This indicated that AZC-induced proteotoxic stress moderately elicited autophagy. However, higher concentration (10 mM) of AZC treatment rather compromised autophagy induction, probably because synthesis of proteins required for

autophagy induction (e.g., Atg8) was also impaired by AZC treatment: protein levels of GFP-Atg8 were remarkably reduced after 10 mM AZC treatment (Fig. 1A). Free GFP was gradually accumulated after AZC treatment (Fig. 1B).

To confirm that the free GFP appearance after AZC treatment is dependent on autophagy, we used cells lacking Atg1, an essential factor for autophagy. Indeed, loss of Atg1 abolished free GFP generation from GFP-Atg8 after AZC treatment, as well as after rapamycin treatment (Fig. 1C). Loss of Atg11, which is required for selective autophagy [17], was not appreciably reduced after rapamycin treatment, indicating that rapamycin treatment dominantly induces bulk (nonselective) autophagy. Similarly, little impairment of AZC-induced autophagy was observed in cells lacking Atg11. This indicated that AZC-induced proteotoxic stress evokes bulk autophagy, like rapamycin treatment.

Nutrient starvation and TORC1 inactivation promote formation of perivacuolar pre-autophagosomal structures (PASs), putative sites producing isolation membranes in budding yeast [16, 17]. Many ATG proteins including Atg8 are recruited at PASs near the vacuole, forms foci upon nutrient starvation and TORC1 inactivation (see Fig. 1D). Consistent with the fact that AZC treatment moderately induced autophagy, this treatment mildly increased PAS formation (Fig. 1D).

Proteotoxic stress attenuates TORC1 activity, but not TORC2 activity, in budding yeast

We suspected that AZC treatment attenuates TORC1 activity, inducing autophagy. TORC1 activity (monitored using the phosphorylation status of Thr737 of Sch9) was drastically lost by rapamycin treatment (Fig. 2A), as described previously [24]. By contrast, Sch9 was partially dephosphorylated after AZC treatment, indicating that TORC1 activity is moderately attenuated by AZC treatment. Consistently, Atg13 was partially dephosphorylated after AZC treatment (Fig. 2B), accounting for autophagy induction. Thus, AZC-induced proteotoxic stress attenuated TORC1 activity, causing Atg13 dephosphorylation and subsequent autophagy induction. In addition to the fact that Atg1 was required for AZC-induced autophagy (Fig. 1C), these findings indicated that the TORC1–Atg13-Atg1 axis mediates AZC-induced autophagy.

TOR protein kinase forms not only TORC1 but also TORC2, which consists of different subunits [25]. TORC2 is insensitive to nutrient starvation and rapamycin, but it is activated and inactivated in response to increased and decreased plasma membrane tension, respectively, to maintain plasma membrane tension [6, 26, 27]. We wondered if proteotoxic stress specifically impairs TORC1. TORC2 activity (monitored using the phosphorylation status of Thr662 of Ypk1) [26] was not fluctuated after AZC treatment (Fig. 6F). These findings indicated that TORC1 activity is specifically attenuated by AZC-induced proteotoxic stress. Collectively, proteotoxic stress attenuates TORC1 activity, but not TORC2 activity

Proteotoixc stress attenuates TORC1 activity and induces autophagy in fission yeast

In the fission yeast *Schizosaccharomyces pombe*, TORC1 regulates autophagy induction via Atg13 phosphorylation in a similar manner [28, 29]. We suspected that proteotoxic stress also attenuates TORC1 activity and induces autophagy in fission yeast. TORC1 activity (monitored using phosphorylation status of Thr415 of Psk1) was completely dephosphorylated after nitrogen starvation (Fig. 3A), as described previously [30]. Although rapamycin treatment does not inhibit cell growth in fission yeast, rapamycin reduces TORC1 activity [31, 32] (Fig. 3A). Similarly, treatment of Torin 1, an inhibitor of TORC1 and TORC2, reduced TORC1 activity (Fig. 3B). AZC treatment partially reduced phosphorylated Psk1 (Figs. 3A, B). Thus, proteotoxic stress also attenuated TORC1 activity in fission yeast. We monitored TORC2 activity using phosphorylation status of Gad8 [33, 34]. Dephosphorylated form of Gad8 was not detected in normal conditions but it appeared after Torin treatment (Fig. B). Gad8 seemingly showed partial dephosphorylation after AZC treatment, although phosphorylated Gad8 was still present. This suggested that proteotoixc stress slightly affects TORC2 activity in fission yeast.

Fission yeast Atg13 was hardly detected in crude extract using an anti-Atg13 antibody [28] in our experimental conditions (Fig. 3A). However, Atg13 was clearly detected after TORC1 inactivation by nitrogen depletion or rapamycin treatment,

although rapamycin treatment impacted on Atg13 to a lesser extent. This suggested that dephosphorylated Atg13 was more detectable in these experimental conditions. When cells were treated with AZC, Atg13 appeared despite to lesser extents as compared with nitrogen starvation (Fig. 3A). This indicated that AZC treatment moderately induces Atg13 dephosphorylation. In addition, the percentage of cells with PAS (monitored using GFP-Atg8) slightly but significantly increased after 6 h of 2.5 mM AZC treatment (Fig. 3C). Thus, proteotoxic stress moderately induced TORC1 inactivation and autophagy.

Proteotoixc stress attenuates mTORC1 activity, but not mTORC2 activity, in human cells

In human cells, it has been reported that AZC-induced proteotoxic stress attenuated mTORC1 activity [10, 11]. However, if AZC treatment induces autophagy via mTORC1 and if it affects mTORC2 are unknown. We confirmed that AZC treatment reduced mTORC1 activity (monitored using phosphorylation statuses of Thr389 of S6K and Ser235/Ser236 of ribosomal protein S6) in human embryonic kidney HEK-293T cells (Fig. 4A). mTORC1 phosphorylates and represses ULK1 kinase (Atg1 homolog) in normal conditions, but ULK1 kinase is dephosphorylated and activated to induce autophagy upon mTORC1 inhibition (e.g., by nutrient starvation or rapamycin treatment)[35]. AZC treatment promoted dephosphorylation of ULK1 (Fig. 4A). Consistently, it has been recently shown that AZC treatment induces autophagy in human cells [36]. By contrast, mTORC2 activity (monitored using phosphorylation status of Ser473 of AKT) was not affected by AZC treatment (Fig. 4A). Thus, AZC-induced proteotoixc stress also attenuated mTORC1 activity, but not mTORC2, in human cells, as well as budding yeast.

Discussion

This study showed that AZC-induced proteotoxic stress attenuated TORC1 activity and induces autophagy via the TORC1–Atg1/ULK1 axis. This system is conserved from yeast to human cells. On the other hand, AZC-induced proteotoxic stress did not affect

TORC2 activity in budding yeast and human cells. TORC1 activity is critical for protein synthesis via promotion of ribosome biogenesis and translation in favorable conditions [37]. Therefore, repression of protein synthesis by TORC1 inactivation in proteotoxic stress conditions might be beneficial for alleviation of accumulation of misfolded proteins [10, 11]. In addition, autophagy induction by TORC1 inactivation in proteotoxic stress conditions should be also adaptive response to eliminate misfolded proteins and protein aggregates [36] (this study).

TORC1 is resident on the vacuole membrane, which is required for TORC1 activation [6]. In budding yeast, upon heat shock TORC1 is inactivated by recruitment to stress granules, ribonucleoprotein granules containing mRNA [24]. However, no such stress granules were formed in budding cells treated with AZC (our unpublished data). This is consistent with the notion that AZC treatment is a suitable condition for induction of only genuine proteotoxic stress. Thus, the molecular mechanism of TORC1 inactivation by AZC-induced proteotoxic stress is different from that of TORC1 inactivation after heat shock stress. In human cells, upon proteotoxic stress (including AZC treatment) the stress-responsive kinase JNK phosphorylates mammalian TOR (mTOR) and its binding partner RAPTOR, causing partial inactivation of mTORC1 [11]. It is an interesting possibility that a similar system is conserved from yeast to human cells.

Dysfunction of proteostasis is related to various diseases including neurodegenerative diseases. This study showed that TORC1 is a conserved key regulator to maintain proteostasis in eukaryotic cells and revealed that yeasts are convenient model organisms for analysis of the role of TORC in response to proteotoxic stress. Since several events caused by TORC1 inactivation in proteotoxic stress conditions remedies proteotoxic stress, pharmacological approach to modulate TORC1 should contribute to alleviation of proteotoxic stress-related diseases.

Materials and methods

Strains and media for budding yeast

The *S. cerevisiae* strains used are listed in Supplementary Table S1. Glucose-containing YPAD (YPD containing 0.01% adenine) and synthetic minimal medium (SD) complemented with the appropriate nutrients for plasmid maintenance were

prepared using standard methods. For assessment of autophagy, when cells harbor plasmids, cells were precultured in SD with the appropriate nutrients, and then cultured in YPAD.

Strains and media for fission yeast

The *S. pombe* strains used are listed in Supplementary Table S2. Synthetic minimal medium, Edinburgh minimal medium (EMM) was prepared as described [38]. A nitrogen-free version (EMM-N) was employed as a starvation medium. Rapamycin and Torin1 were purchased from Millipore (#55211, Billerica, MA) and Tocris bioscience (#4247, Bristol UK), respectively.

Human cells and media

HEK293T cells were obtained from the American Type Culture Collection (ATCC) and cultured in 4.5 g/L glucose Dulbecco's Modified Eagle's Medicum (DMEM) (Sigm-Aldrich, #D5671) supplemented with 10% fetal bovine serume (Gibco # 10500064), 4 mM L-glutamine (Sigm-Aldrich, #G7513), 1 mM sodium pyruvate (Sigm-Aldrich, #S8636), and 1 x penicillin/streptomycin (Sigm-Aldrich, #P4333). AZC was dissolved in PBS.

Protein extraction from budding yeast

Proteins were extracted by a post-alkaline extraction method in accordance with a previous report [20]. Briefly, cells (10 ml culture, OD_{600} = 0.2-0.8) were treated with 200 µl of 0.1 M NaOH for 5 min and then the pellet was collected by centrifugation. The pellet was resuspended in sample buffer (60 mM Tris-HCl (pH 6.8), 5% glycerol, 2% SDS, 4% 2-mercaptoethanol and 0.0025% bromophenol blue) at 95°C for 5 min. Crude extracts were cleared by centrifugation and the supernatant was used for western blotting analysis.

Protein extraction from fission yeast

Proteins extraction was performed in accordance with a previous report [30]. Briefly, cell cultures (7 ml, OD_{600} = 0.5-0.8) were mixed with trichloroacetic acid at a final concentration of 7% and put on ice at least for 5 min. The pellet was then washed twice

with cold acetone and dried. The cells were disrupted in lysis buffer for fission yeast [30] with glass beads and then suspended in sample buffer at 94°C for 6 min. Crude extracts were cleared by centrifugation and the supernatant was used for western blotting analysis.

Protein extraction from human cells

After AZC treatment, cells were washed twice with ice-cold PBS and lysed with SDS sample buffer. Crude extracts were boiled at 95 ºC for 5 min and used for western blotting analysis.

Immunoblotting analysis

Immunoblotting and detection of proteins extracted from budding yeast [39] and fission yeast [30] were performed in accordance with previous reports. Human cellular proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, #10500003), The membranes were incubated with Odyssey blocking buffer (LI-COR, #927-50000, Lincoln, NE), followed by incubation with primary antibodies for overnight. The membranes were washed three times with TBST and incubated with IRDye 800CW coupled with anti-rabbit IgG (LI-COR, #926-3221) or anti-mouse IgG (LI-COR, #926-32210). The infrared signals were detected by an Odyssey Fc (LI-COR). Information of antibodies used in this study is shown in Supplementary Table S3. All western blotting experiments were performed at least twice independently to confirm reproducibility of the results.

Microscope observations

Cell and GFP images in budding and fission yeast were captured using an Axio Imager M1 microscope with a cooled CCD camera (Carl Zeiss AxioCam MRm) and a BZ-9000 microscope (Keyence Corporation, Osaka, Japan), respectively. For examination of PAS formation, more than 100 cells were counted and were scored. All microscope observations were performed at least three independently to confirm reproducibility of the results. Data are shown as averages ± SDs.

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Author contributions

TU, MS and AN designed the experiments. KS mainly conducted the experiments. AK, MS and AN performed some experiments. TM provided invaluable materials and helped to design the experiments. MNH supervised some experiments. TU mainly wrote the paper. MS and AN helped to write the paper.

Competing Interests

None.

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Figure legends

Figure 1. AZC-induced proteotoxic stress elicits autophagy in budding yeast

(A) Exponentially growing budding yeast cells of wild-type strain BY4741 harboring plasmid pSCU1998 (pRS416-GFP-ATG8) were treated with 200 ng/ml rapamycin or 2.5, 5 or 10 mg/ml AZC for 3 h. Whole cell extracts were subjected to immunoblotting using an anti-GFP antibody. Cyclin-dependent kinase (CDK) was detected as a loading control using an anti-CDK antibody. (B) Cells of strain BY4741 harboring plasmid pSCU1998 (pRS416-GFP-ATG8) were treated with 200 ng/ml rapamycin or 2.5 mg/ml AZC for indicated times. Whole cell extracts were subjected to immunoblotting using an anti-GFP antibody. Pgk1 was detected as a loading control using an anti-Pgk1 antibody. (C) Cells of strains BY4741 (wild-type), SCU3365 (*atg1*Δ) and SCU3464 (*atg11*Δ) harboring plasmid pSCU1998 (pRS416-GFP-ATG8) were treated with rapamycin or AZC for 4 h. Whole cell extracts were subjected to immunoblotting. (D) Cells of strain US356 (wild-type) harboring plasmid pSCU1998 (pRS416-GFP-ATG8) were treated with rapamycin or AZC for indicated times. Cell images with GFP signals were captured using a fluorescence microscope. Arrowheads indicate perivacuolar GFP-Atg8 puncta. Scale bars, 5 μm. Cells with GFP-Atg8 puncta were counted and are expressed as percentages (averages ± SDs).

Figure 2. AZC-induced proteotoxic stress attenuates activity of TORC1, but not of TORC2, in budding yeast

(A) Cells of strain SCU2959 (*3HA-SCH9*) were treated with 2.5 mg/ml AZC for indicated times. Whole cell extracts were subjected to western blotting using an anti-phospho-Sch9 (T737) antibody. Total amounts of Sch9 were detected using an anti-HA antibody. Cells treated with 200 ng/ml rapamycin for 15 min were used for the control. P-Sch9, phosphorylated Sch9. (B) Cells of wild-type strain BY4741 harboring plasmid pSCU1875 (pATG13) were treated with AZC for indicated times. Whole cell extracts were subjected to western blotting using an anti-Atg13 antibody. For detection of the phosphorylation statuses of Atg13, 7.5% acrylamide gels were used. Cells treated with rapamycin for 15 min were used for the control. P-Atg13, phosphorylated Atg13. (C) Cells of strain SCU5513 (*YPK1-GFP*) were treated with AZC for indicated times. Whole cell extracts were subjected to western blotting using an anti-phospho-Ypk1 (T662). Total amounts of Ypk1 were detected using the anti-GFP antibody. P-Ypk1, phosphorylated Ypk1.

Figure 3. Proteotoixc stress attenuates TORC1 activity and induces autophagy in fission yeast

(A) Exponentially growing fission yeast cells of wild-type strain L972 were diluted in fresh EMM medium and further incubated for 3 h at 30ºC. Cells were treated with 200 nM rapamycin, or 1, 2.5 mM AZC for 4 h. An aliquot was washed and starved with EMM-N for 4h (-N). Whole cell extracts were subjected to immunoblotting using anti-phospho-p70 S6K (Thr389) and anti-Atg13 antibodies. α-tubulin was detected as a loading control using an anti-α-tubulin antibody. (B) Cells of a strain AN0175 (*gad8*$^{+}$-*3HA*) were diluted and further incubated as described in (A). Cells were treated with 3 μM Torin 1, or 1, 2.5 mM AZC for 4 h. Whole cell extracts were subjected to immunoblotting using anti-phospho-p70 S6K (Thr389) and anti-HA antibodies. P-Gad8, phosphorylated Gad8. (C) Cells of strain JT268 (*GFP-atg8*$^{+}$) were treated with 2.5 mM AZC or 200 nM rapamycin, or transferred to EMM-N for 6 h as described in (A). Cell and GFP images were captured using a fluorescence microscope. Arrowheads indicate GFP-Atg8 puncta. Scale bars, 10 μm.Cells with GFP-Atg8 puncta were counted and are expressed as percentages (averages ± SDs). The *p*-values were calculated using two-tailed Student's *t*-test.

Figure 4. Proteotoixc stress attenuates mTORC1 activity, but not mTORC2 activity, in human cells

HEK-293T cells were treated with 5 mM AZC for 3 or 6 h. Whole cell extracts were subjected to western blotting analysis using the corresponding antibodies. Actin serves as a loading control.