Dispersion of Endoplasmic Reticulum-associated Compartments by 4-phenyl Butyric Acid in Yeast Cells

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ABSTRACT. In yeast *Saccharomyces cerevisiae* cells, some aberrant multimembrane-spanning proteins are not transported to the cell surface but form and are accumulated in endoplasmic reticulum (ER)-derived subcompartments, known as the ER-associated compartments (ERACs), which are observed as puncta under fluorescence microscopy. Here we show that a mutant of the cell surface protein Pma1, Pma1-2308, was accumulated in the ERACs, as well as the heterologously expressed mammalian cystic fibrosis transmembrane conductance regulator (CFTR), in yeast cells. Pma1-2308 and CFTR were located on the same ERACs. We also note that treatment of cells with 4-phenyl butyric acid (4-PBA) compromised the ERAC formation by Pma1-2308 and CFTR, suggesting that 4-PBA exerts a chaperone-like function in yeast cells. Intriguingly, unlike ER stress induced by the canonical ER stressor tunicamycin, ER stress that was induced by Pma1-2308 was aggravated by 4-PBA. We assume that this observation demonstrates a beneficial aspect of ERACs, and thus propose that the ERACs are formed through aggregation of aberrant transmembrane proteins and work as the accumulation sites of multiple ERAC-forming proteins for their sequestration.

Key words: protein aggregation, organelle, unfolded protein response, ER stress, 4-PBA

Introduction

The endoplasmic reticulum (ER) is a membrane-bound cellular compartment in which newly synthesized secretory and transmembrane proteins are folded before being transported to the cell surface or other organelles. As exemplified by the vertebrate chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) and its mutants, misfolded ER-client proteins are retained in the ER and are thus unable to work normally (Cheng *et al.*, 1990). Moreover, misfolded proteins *per se* are harmful to cells when abundantly accumulated in the ER lumen (Umebayashi *et al.*, 1997). Dysfunctional situations in the ER are tightly linked to ER accumulation of misfolded proteins and is collectively called ER stress.

The unfolded protein response (UPR) is a cytoprotective gene-induction program that triggered upon ER stress (Ron and Walter, 2007; Mori, 2009). It is widely believed that in *Saccharomyces cerevisiae* (hereafter simply called yeast) cells, the ER-located transmembrane protein Ire1 acts as a sole ER-stress sensor that evokes the UPR. Ire1 senses ER stress via multiple mechanisms (Kimata *et al.*, 2007; Kimata and Kohno, 2011; Ishiwata-Kimata *et al.*, 2018), and works as an endoribonuclease that promotes splicing of the *HAC1* mRNA. The spliced form of the *HAC1* mRNA is translated into a nuclear transcription-factor protein that is responsible for transcriptional induction in the UPR.

Misfolded proteins often form foci in cells, possibly in order to be sequestered from other cellular components. For instance, according to Escusa-Toret *et al.* (2013), misfolded cytosolic proteins are accumulated on the ER surface to form puncta called the Q bodies, the formation of which is thought to be beneficial for yeast cells. Some previous publications have reported that misfolded multimembrane-spanning proteins form ER-associated punctate structures, which is called the ER-associated compartments (ERACs), in yeast cells (Huyer *et al.*, 2004; Liu *et al.*, 2006; Fu and Sztul, 2009; Kakoi *et al.*, 2013). As well as mutant versions of yeast endogenous transmembrane proteins, such as Ste6, heterologously expressed CFTR forms the ERAC even when not carrying a mutation.

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Abbreviations: 4-PBA, 4-phenyl butyric acid; carboxy-DCFDA, 5-(and-6-)carboxy-2',7'-dichlorofluorescein diacetate; CFTR, cystic fibrosis transmembrane conductance regulator; DTT, dithiothreitol; ER, endoplasmic reticulum; ERAC, ER-associated compartment; HA, hemagglutinin; PAGE, poly-acrylamide gel electrophoresis; PI, propidium iodide; SD, synthetic dextrose; TCA, trichloroacetic acid; UPR, unfolded protein response; YGRC, Yeast Genetic Resource Center.

While some previous publications addressed the nature of the ERACs (Huyer *et al.*, 2004; Fu and Sztul, 2009; Kakoi *et al.*, 2013), many questions remain unanswered about their physiological properties and relevance. For instance, is the ERAC formation beneficial for cells? Are different ERAC-forming proteins accumulated in the same ERACs? Moreover, the molecular mechanism for ERAC formation is still largely unknown.

For better understanding of the ERACs, in this study, we explore yeast cells expressing a mutant form of Pma1, which is a plasma membrane-located multimembrane-spanning protein that acts as a proton pump when not carrying a mutation (Serrano *et al.*, 1986). This mutant Pma1, which we named as Pma1-2308, formed the ERACs and induced the UPR when highly expressed in yeast cells. Cellular treatment with the chemical chaperone 4-phenyl butyric acid (4-PBA) inhibited the ERAC formation of Pma1-2308, leading to aggravation of ER stress caused by this mutant protein. This observation argues for a beneficial aspect of the ERACs.

Materials and Methods

Plasmid construction

Plasmid pTH761-CEN-mCherry_v4 contains a yeast codon-optimized version of the mCherry cDNA (Chu *et al.*, 2014). The human CFTR cDNA was a gift from Prof. Kenji Kohno (Nara Inst. Sci. Tech.). Using these cDNAs as templates, we obtained the mCherry-coding and the CFTR-coding DNA fragments through high-fidelity PCR with Pyrobest DNA polymerase (Takara). The Pma1-coding DNA fragment was PCR-amplified from a yeast genome using conventional Takara ExTaq DNA polymerase.

The yeast centromeric plasmid pPM28 (*URA3* marker; Merksamer *et al.*, 2008) was used for expression of eroGFP under control of the *TDH3* plasmid. After *NheI*/*XbaI* digestion, which resulted in excision of the eroGFP-coding sequence, pPM28 was ligated with the Pma1-coding and the mCherry-coding DNA fragments or the CFTR-coding and the mCherry-coding DNA fragments. The resulting plasmids, pYT-TDH3p-PMA1-mCherry and pYT-TDH3p-CFTR-mCherry, respectively, were used for expression of wild-type Pma1-mCherry and CFTR-mCherry in yeast cells. Due to PCR error, we also obtained the *PMA1-2308* mutant version of pYT-TDH3p-PMA1-mCherry, which is named pYT-TDH3p-PMA1-2308-mCherry. The *CUP1* promoter sequence was PCR-amplified from the yeast genome and was substituted on the *TDH3* promoter of pYT-TDH3p-PMA1-2308-mCherry for generation of pYT-CUP1p-PMA1-2308-mCherry, which was used for expression of Pma1-2308-mCherry under the control of the *CUP1* promoter in yeast cells. The 13-tandem-copy Myc epitope-coding sequence was PCR-amplified from the plasmid pFA6a-13Myc-kanMX6 (Bahler *et al.*, 1998), and was substituted on the mCherry-coding sequence of pYT-CUP1p-PMA1-2308-mCherry

for generation of pYT-CUP1p-PMA1-2308-Myc, which was used for expression of Pma1-2308-Myc under the control of the *CUP1* promoter in yeast cells. These plasmids were deposited in the Yeast Genetic Resource Center (YGRC), and the plasmid sequence information is available in our dropbox site (<https://www.dropbox.com/sh/ocqptzlcfa7t0/AACA61a-YeaTkJSigzmJbUhwa?dl=0>) and on the YGRC website (<http://yeast.nig.ac.jp/yeast/top.shtml>). For the empty vector control, pRS316 (Sikorski and Hieter, 1989) was employed.

The hemagglutinin (HA) epitope-tagged Ire1-gene centromeric (single-copy) plasmid pRS315-IRE1-HA, *IRE1*-gene centromeric (single-copy) plasmid pRS313-IRE1 and the empty vector pRS313 (*HIS3* marker) were described in Kimata *et al.* (2004) and Sikorski and Hieter (1989).

Yeast strains

Unless otherwise noted, BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was used as a wild-type strain and transformed with the aforementioned plasmids. In the experiments using 4-PBA, KMY1516 (*MATa his3 leu2 lys2 ura3 trp1 ire1::TRP1 leu2::UPRE-GFP::LEU2 lys2::UPRE-lacZ::LYS2*; Kimata *et al.*, 2004) carrying pRS313-IRE1 was employed as a wild-type strain and transformed with the aforementioned plasmids, because the viability of BY4742 was somewhat sensitive to 4-PBA (data not shown). For the *IRE1* gene-knockout experiments, KMY1516 cells transformed with pRS313-IRE1 and pRS313, respectively, were used as the *IRE1*⁺ and the *ire1Δ* strains. For cellular expression of Ire1-HA, we employed KMY1015 (*MATa his3 leu2 lys2 ura3 trp1 ire1::TRP1*; Mori *et al.*, 1996) transformed with pRS315-IRE1-HA.

Transformation of the BY4742 cells with *PvuI*-digested pYT-CUP1p-PMA1-2308-mCherry together with *NotI*-digested pRS315 (*LEU2* marker; Sikorski and Hieter, 1989) yielded the *LEU2*-marker version of pYT-CUP1p-PMA1-2308-mCherry via *in vivo* homologous recombination. The resulting transformants were again transformed with pPM28 in order to obtain cells producing both Pma1-2308-mCherry and eroGFP.

BY4742-derived *HRD1/DOA10*-double knockout strain (*hrd1::kanMX4 doa10::SpHIS5MX*) has been described previously (Mai *et al.*, 2018), and here was transformed with pYT-TDH3p-CFTR-mCherry. Alternatively, this *HRD1/DOA10*-double knockout strain was modified to carry both the *LEU2*-marker version of pYT-CUP1p-PMA1-2308-mCherry and the mCherry-to-GFP replaced version of pTDH3p-CFTR-mCherry, which were also generated through *in vivo* homologous recombination.

Yeast transformation was performed using the standard lithium-acetate-based protocol (Kaiser *et al.*, 1994).

Yeast culture

Unless otherwise noted, yeast cells were aerobically and exponentially shaken in synthetic dextrose (SD) medium containing 2% glucose, 0.66% Difco Yeast Nitrogen Base (w/o amino acid) and appropriate auxotrophic requirements at 30°C. Alternatively, cells

were cultured at 23°C to obtain brighter CFTR-mCherry or CFTR-GFP signals. SD medium plates containing 2% agar were incubated at 30°C after inoculation of yeast cells. For chemical treatment of cells, tunicamycin (Sigma-Aldrich; 2 mg/mL in dimethyl sulfoxide for stock solution), CuSO₄ (0.5 M in water for stock solution), and 4-PBA (Sigma-Aldrich; 0.5 M in water for stock solution) were added into cultures, which were further shaken at 30°C.

Protein and RNA analyzes

As described previously (Kimata *et al.*, 2003; Mai *et al.*, 2018), cells were lysed in the presence of 1% triton X-100 and analyzed by Western blotting using mouse monoclonal antibodies 12CA5 (anti-HA epitope; Roche) and 9E10 (anti-c-Myc epitope anti-HA antibody (anti-c-Myc; Roche). Cell lysates were centrifuged at 15,000×g for 30 min to obtain pellet fractions. In order to denature proteins, we added 10% trichloroacetic acid (TCA) and 100 mM dithiothreitol (DTT) into the SDS poly-acrylamide gel electrophoresis (SDS-PAGE) loading buffer.

Splicing of the *HAC1* mRNA was monitored by RT-PCR amplification of the *HAC1* species from total RNA samples, which was followed by standard agarose-gel electrophoresis (Promlek *et al.*, 2011; Mai *et al.*, 2018). The ethidium bromide-fluorescence images of the resulting gels was used for image analysis which was followed by calculation of “the *HAC1* mRNA-splicing efficiency” using the formula $[100 \times (\text{band intensity of } HAC1^i) / \{(\text{band intensity of } HAC1^i) + (\text{band intensity of } HAC1^u)\}]$, where *HAC1ⁱ* and *HAC1^u*, respectively, are the spliced and unspliced forms of the *HAC1* mRNA.

Microscopic observation

In order to stain cells with 5-(and-6-)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA), cells were suspended in sodium citrate buffer (pH 5.0) containing 2% glucose and 10 mM carboxyl-DCFDA for 30 min.

We observed yeast cells under the Keyence BZ-9000E microscope and the objective lens CFI Plan Apo λ100xH (Nikon). GFP and carboxy-DCFDA fluorescence was observed using a preset filter set (excitation wavelength, 470/30; dichroic mirror wavelength, 495; emission, 535/25). The mCherry fluorescence was observed using another preset filter set (excitation wavelength, 560/20; dichroic mirror wavelength, 595; emission, 630/30). The exposure time was 0.5 or 1.0 sec for wild-type Pma1-mCherry or Pma1-2308-mCherry fluorescence, 1.0 sec for eroGFP fluorescence, 8 sec for CFTR-mCherry fluorescence and 0.8 sec for carboxy-DCFDA fluorescence.

In order to quantitatively express ER dispersion of Pma1-2308-mCherry, we counted cells showing nuclear-ER unbroken ring-like fluorescent image of Pma1-2308-mCherry. More than 50 fluorescence-emitting cells were checked per sample, and its portion against total fluorescence-emitting cells are expressed as the mean of three independent cultures.

Flow cytometry

The flow cytometer Accuri C6 Plus (BD Biosciences) were operated under the following conditions: flow rate, slow; threshold, FCS-H>80,000; count cell number, 20,000. In order to count propidium iodide (PI)-stainable cells, cultures were mixed with PI (10 µg/mL (final concentration)), further incubated for 30 min and subjected to flow cytometry.

Statistical analysis

Numerical data were obtained through multiple (basically three) determinations using independent clones, and were evaluated by the unpaired (homoscedastic) two-tailed t-test.

Results

At the beginning of this study, we constructed a yeast expression plasmid (pYT- TDH3p-PMA1-mCherry) to produce a C-terminally mCherry-tagged version of Pma1 (Pma1-mCherry) under the control of the strong and constitutive *TDH3* promoter. As shown in Fig. 1A and Fig. S1, mCherry fluorescence was observed in the cell periphery and the vacuoles, which were stained by the vacuole-staining dye carboxy-DCFDA, of yeast cells harboring this plasmid. This fluorescence pattern is consistent with that reported by Eastwood and Meneghini (2015) and suggests that Pma1-mCherry on the plasma membrane was partly subjected to endocytosis and transported to the vacuoles.

Since we yielded the *PMAL* gene through PCR in which the DNA replication fidelity was low (see the Materials and Methods section) to construct pYT-TDH3p-PMA1-mCherry, we also obtained a *PMAL* gene-mutant version of this plasmid. Based on our further analysis described in the next paragraph, we named this mutant as *PMAL*-2308. Expression of Pma1-2308 is likely to harm cells since yeast cells transformed with the *PMAL*-2308 mutant variant of pYT-TDH3p-PMA1-mCherry (pYT-TDH3p-PMA1-2308-mCherry) proliferated very slowly (Fig. S2). Intriguingly, unlike wild-type Pma1-mCherry, Pma1-2308-mCherry exhibited a punctate distribution pattern, which did not merge with the vacuole marker, in yeast cells (Fig. 1A and Fig. S1).

The *PMAL*-2308 mutant carried one silent (non-amino acid-replacing) nucleotide replacement (Thymine1548-to-Cytosine) and four non-silent (amino acid-replacing) nucleotide replacements which are indicated in Fig. S3A (the nucleotides are numbered from the initiation codon of the *PMAL* gene). In order to determine which is responsible for the punctum-forming phenotype of the *PMAL*-2308 mutation, we introduced each of the non-silent nucleotide replacements into the wild-type Pma1-mCherry gene. As shown in Fig. S3A, Pma1-mCherry exhibited a punctate distribution when carrying the Thymine 2308-to-Cytosine

Fig. 1. Pma1-2308 forms the ERACs. (A) Wild-type cells producing wild-type Pma1-mCherry or Pma1-2308-mCherry under *TDH3*-promoter control were observed under fluorescence microscopy. (B) Wild-type cells that produce Pma1-2308-mCherry under *CUP1*-promoter control were cultured with or without 500 μ M CuSO_4 for 4 hr and observed under fluorescence microscopy. (C) Wild-type cells that produce both (or either) Pma1-2308-mCherry (under *CUP1*-promoter control) and eroGFP (under *TDH3*-promoter control) were cultured with 500 μ M CuSO_4 for 4 hr and observed under fluorescent microscopy.

Fig. 2. Pma1-2308 and CFTR co-localize in the same ERACs. *hrd1Δdoa10Δ* cells that produce both Pma1-2308-mCherry (under *CUP1*-promoter control) and CFTR-GFP (under *TDH3*-promoter control) were cultured with 500 μ M CuSO_4 for 4 hr and observed under fluorescent microscopy.

single mutation. It should be also noted that the Thymine 2308-to-Cytosine single replacement was also responsible for the growth-retardation phenotype of the *PMA1-2308* mutation (Fig. S3B).

In order to grow yeast cells carrying the *PMA1-2308* gene to an extent that sufficiently allowed the below-mentioned experiments, we constructed yeast cells in which Pma1-mCherry-2308 was expressed from an inducible promoter. As expected, yeast cells transformed with pYT-CUP1p-PMA1-2308-mCherry, from which expression of Pma1-2308-mCherry was regulated under the control of the copper ion-inducible *CUP1* promoter, exhibited fluorescence when cultured in the presence of 500 μ M CuSO_4 (Fig. 1B). According to our flowcytometric analysis (Fig. S4), the expression level of Pma1-2308-mCherry reached a maximum level 4-hr after induction onset under our experimental condition.

In the experiment shown in Fig. S5, the C-terminally Myc-epitope-tagged versions of wild-type Pma1 (wild-type Pma1-Myc) and Pma1-2308 (Pma1-2308-Myc) were expressed in wild-type cells, the lysates of which were analyzed by anti-Myc Western blotting. Although protein samples were denatured by SDS, DTT and TCA before electrophoresis (see the Materials and Methods section), Pma1-2308-Myc, as well as wild-type Pma1-Myc, appeared as slightly ladderized and smeared bands for unknown reason(s) (Fig. S5). We next checked stability of

wild-type Pma1-Myc and Pma1-2308-Myc using the cycloheximide-chase technique. According to our data shown in Fig. S6, Pma1-2308-Myc seemed to be fairly stable (more stable than wild-type Pma1-Myc) in yeast cells.

In the experiment shown in Fig. 1C, we observed yeast cells expressing both (or either) Pma1-2308-mCherry and eroGFP, which is an ER-localized variant of GFP (Merksamer *et al.*, 2008). When expressed alone in yeast cells, eroGFP showed a typical double-ring ER distribution pattern. Meanwhile, eroGFP was also distributed to punctate dots that were located on or adjacent to the double rings when it was expressed together with Pma1-2308-mCherry. It should be also noted that Pma1-2308-mCherry was located on the eroGFP puncta. We thus deduce that Pma1-2308-mCherry forms and is accumulated in the ERACs, as described in the Discussion section.

As described in the Introduction section, heterologously expressed CFTR is also located in the ERACs in yeast cells. In order to observe intracellular localization of C-terminally GFP-tagged CFTR (CFTR-GFP), we employed here the *hrd1Δdoa10Δ* double deletion mutant strain because CFTR-GFP is quickly degraded through the ER-associated protein degradation in a manner dependent on the E3 enzymes Hrd1 and Doa10 (Gnann *et al.*, 2004) and hardly emits observable fluorescence in wild-type yeast cells. As shown in Fig. 2, CFTR-GFP and Pma1-2308-mCherry were co-localized, indicating that these two different ERAC-forming proteins are accumulated in the same ERACs.

Next, we asked if cellular expression of Pma1-2308-mCherry induces ER stress that triggers the UPR, which is monitorable through the *HAC1* mRNA splicing. As described previously (Promlek *et al.*, 2011; Mai *et al.*, 2018), here we amplified the *HAC1* splices from total RNA samples using the reverse transcription (RT)-PCR technique. Fig. 3A shows that Pma1-2308-mCherry, but not wild-type Pma1-mCherry, triggered *HAC1*-mRNA splicing when it was expressed from the *TDH3* promoter. According to our data shown in Fig. S7, the Thymine 2308-to-Cytosine single replacement was responsible for the ER-stressing phenotype of the *PMA1-2308* mutation.

UPR induction by the Pma1-2308-mCherry expressed from the *CUP1* promoter is described later in this paper. In the experiment shown in Fig. 3B, cells were grown on agar media containing or not containing an inducible concentration of CuSO_4 . Cells carrying the *CUP1* promoter-controlled Pma1-2308-mCherry-expression plasmid showed growth retardation, which was aggravated by the absence of the *IRE1* gene, on the inducer-containing plate. These observations indicate that, at least partly, Pma1-2308-mCherry harms yeast cells through induction of ER stress. Fig. S8 exhibits the ERAC formation of Pma1-2308-mCherry in *ire1Δ* cells.

Since it is widely accepted that 4-PBA is a chemical chaperone that inhibits protein aggregation (Kubota *et al.*,

Fig. 3. Pma1-2308 induces the UPR. (A) Wild-type cells producing either (or neither) wild-type Pma1-mCherry or Pma1-2308-mCherry under the *TDH3*-promoter control were checked for *HAC1* mRNA-splicing efficiency. ****: $p < 0.001$, n.s.: not significant ($p > 0.05$). (B) *IRE1+* and *ire1Δ* cells carrying the *CUP1* promoter-controlled Pma1-2308-mCherry expression plasmid or the empty vector were spotted on SD agar plates supplemented or not with 50 μ M CuSO_4 , which were then incubated for 3 days. On each spot, cells equivalent to $\text{OD}_{600} = 0.00015$ were inoculated.

2006; Kolb *et al.*, 2015), we asked if 4-PBA affects the cellular localization of ERAC-forming proteins. As shown in Fig. 4A and 4B, treatment of cells with 1 mM 4-PBA caused dispersed distribution of Pma1-2308-mCherry and C-terminally mCherry-tagged CFTR (CFTR-mCherry). Since the cellular distribution of Pma1-2308-mCherry and CFTR-mCherry in 4-PBA-treated cells demonstrated a typical double-ring-like ER pattern, we deduce that, at least partly, they are retained in the ER without being transported to the cell surface.

In the experiment shown in Fig. 4C, lysates of cells producing Pma1-2308-Myc were subjected to anti-Myc Western blotting, indicating that treatment of cells with 4-PBA did not significantly change the cellular abundance of Pma1-2308-Myc (compare Lane 2 to Lane 1). We also subjected the total cell lysates to high-speed centrifugation and noticed that cellular treatment with 4-PBA decreased the abundance of Pma1-2308-Myc in the pellet fractions (compare Lane 4 to Lane 3).

Next, we asked if 4-PBA can disperse already-ERAC-forming Pma1-2308-mCherry proteins. In the experiment shown in Fig. 4D, yeast cells producing Pma1-2308-mCherry from the *CUP1* promoter through 4-hr culture with 500 μ M CuSO_4 were washed with normal medium and further cultured in the absence of an inducible concentration of CuSO_4 (chase culturing). We then found that Pma1-2308-mCherry was dispersed, though not completely, when chase culturing was performed in the presence of 1 mM 4-PBA. In order to express this observation quantitatively, we counted cells showing dispersed distribution of Pma1-2308-mCherry, which was represented by its nuclear-ER unbroken ring-like image (Fig. 4D).

In the final part of this study, we asked if ER stress is

alleviated or aggravated when the ERAC formation of Pma1-2308-mCherry is compromised by cellular treatment with 4-PBA. According to our previous publication (Mai *et al.*, 2018), the cellular abundance of C-terminally HA epitope-tagged Ire1 (Ire1-HA) was drastically decreased by treatment of yeast cells with 5 mM 4-PBA. However, such was not observed, and the cellular Ire1-HA abundance was almost unchanged when yeast cells were cultured in the presence of 1 mM 4-PBA for 1 hr (Fig. 5A). In order to monitor the effect of 4-PBA on the cellular UPR level, we thus employed the experimental condition represented in Fig. 5B, in which yeast cells were treated with 1 mM 4-PBA for no longer than 1 hr (4-PBA was added 3-hr after addition of 500 μ M CuSO_4 into culture). Fig. 5C shows that, under this condition, Pma1-2308-mCherry was partially dispersed. Notably, 4-PBA further increased the UPR level that was induced by Pma1-2308-mCherry, at least under the experimental procedure employed here (Fig. 5D). We therefore deduce that the dispersion of Pma1-2308-mCherry by 4-PBA aggravates ER stress. This observation is in contrast with the case of stressing yeast cells with the canonical ER stressor tunicamycin, in which 4-PBA compromised the UPR (Fig. S9). In the experiments shown in Fig. 5, we avoided treatment of yeast cells with 4-PBA for a longer duration, which decreased the cellular Ire1 abundance (data not shown), possibly leading to a reduction of the *HAC1*-mRNA level and to an underestimation of the cellular ER-stress level. PI is known to stain dead yeast cells (Kwolek-Mirek and Zadrąg-Tecza, 2014). In the experiment shown in Fig. 5E, we measured the portion of PI-stainable cells in cultures and found that the cellular expression of Pma1-2308-mCherry caused a reduction of cell viability, which was aggravated by 4-PBA.

Fig. 4. 4-PBA compromises the ERAC formation. (A) Wild-type cells that produce Pma1-2308-mCherry under *CUP1*-promoter control were cultured with 500 μM CuSO₄ and 1 mM 4-PBA (or with 500 μM CuSO₄ only) for 4 hr and observed under fluorescence microscopy. (B) *hrd1Δdoa10Δ* cells producing CFTR-mCherry under *TDH3*-promoter control were cultured with or without 500 μM 4-PBA for 3 hr and observed under fluorescence microscopy. (C) Wild-type cells that produce Pma1-2308-Myc under *CUP1*-promoter control were cultured with 500 μM CuSO₄ and 1 mM 4-PBA (or with 500 μM CuSO₄ only) for 4 hr and lysed in the presence of 1% Triton-X100. The total cell lysates were then separated by high-speed centrifugation and analyzed by anti-Myc Western blotting. Each lane contained sample from cells equivalent to OD₆₀₀=0.13 for “total lysates” and from cells equivalent to OD₆₀₀=2.5 for “pellets.” Anti-Pgk1 Western blot served as a loading control. *: $p < 0.05$, n.s.: not significant ($p > 0.05$). (D) After being cultured in the presence of 500 μM CuSO₄, cells producing Pma1-2308-mCherry under *CUP1*-promoter control were washed four times with SD medium (CuSO₄ washout) and further incubated in the presence or absence of 1 mM 4-PBA for 150 min (chase culture).

Discussion

To our knowledge, the ERAC formation in yeast cells was initially reported by Hoyer *et al.* (2004). The ERACs are

defined as ER subcompartments in which misfolded multimembrane-spanning proteins are localized. The ER is a flat-shaped membranous sac, which, according to the electron microscopic images reported by Hoyer *et al.*

Fig. 5. 4-PBA enhances the UPR induced by Pma1-2308-mCherry. (A) Cells producing Ire1-HA were treated with 1 mM 4-PBA for 1 hr or left untreated, and the total lysates from cells equivalent to $\text{OD}_{600}=0.13$ were analyzed by anti-HA Western blotting. Anti-Pgk1 Western blot served as a loading control. (B) Procedure for treatment of cells with 4-PBA 3 hr after the induction onset of Pma1-2308-mCherry expression. (C) Cells that produce Pma1-2308-mCherry under *CUP1*-promoter control were treated as shown in panel B, and were observed under fluorescence microscopy. (D) and (E) Cells that produce Pma1-2308-mCherry under *CUP1*-promoter control were treated as shown in panel B (or cultured with either or none of the chemicals), and were checked for the *HAC1*-mRNA splicing (D) or the portion of PI-stainable cells in the cultures (E). *: $p < 0.05$, n.s.: not significant ($p > 0.05$).

(2004) and Fu and Sztul (2009), is partly folded and stacked to form multiple layers on the site where the ERACs are formed. This observation is consistent with our fluorescence microscopy images of yeast cells producing both eroGFP, which is thought to be diffusively located in the ER, and Pma1-2308-mCherry (Fig. 1C). In yeast cells producing both fluorescent proteins, eroGFP fluorescence displayed faint double rings and bright puncta adjacent to the rings, and Pma1-2308-mCherry was located on the eroGFP puncta. It should be also noted that Pma1-2308-mCherry and heterologously expressed CFTR were co-localized (Fig. 2). To our knowledge, this is the first report showing that two different ERAC-forming proteins are accumulated in the same ERACs.

As described in Kubota *et al.* (2006), 4-PBA is likely to function as a chemical chaperone that prevents aggregation of denatured proteins. Moreover, 4-PBA suppresses the UPR in mammalian and yeast cells, and is thought to be a promising chemical that can be used for therapeutic purposes for various diseases caused by ER accumulation of misfolded proteins (Kubota *et al.*, 2006; Kolb *et al.*, 2015).

However, we reported previously that at least in yeast cells, 4-PBA compromises the UPR not via restoration of the protein folding status in the ER but via reduction of the cellular Ire1 abundance. Meanwhile, here we employed a lower concentration of 4-PBA than that was used in previous publications by us and others (Kubota *et al.*, 2006; Mai *et al.*, 2018), and found that 4-PBA suppresses the tunicamycin-induced UPR without reduction of the cellular Ire1 abundance (Fig. 5A and Fig. S9). Intriguingly, 4-PBA extinguished the ERACs and caused a dispersed distribution of ERAC-forming proteins across the ER (Fig. 4). Therefore, we conclude that 4-PBA can actually work as a molecular chaperone in yeast cells.

In the experiment shown in Fig. 4C, we separated cell lysates through high-speed centrifugation, and found that the amount of Pma1-2308-Myc in the pellet fraction was decreased by cellular treatment with 4-PBA. We deduce that accumulation of Pma1-2308-Myc in the pellet fraction, which was compromised by 4-PBA, represents its aggregation. Taken together, our observations support the notion that the ERACs are formed through aggregation of ERAC-

forming proteins. In agreement with this idea, the fluorescence-recovery-after-photobleaching analysis performed by Fu *et al.* (2009) showed that in yeast cells, the CFTR becomes less mobile when accumulated into the ERACs.

Unlike other ERAC-forming proteins (Kakoi *et al.*, 2013 and our unpublished data), cellular expression of Pma1-2308-mCherry clearly induced the UPR in yeast cells (Fig. 3A and Fig. 5D), indicating that Pma1-2308-mCherry damages the ER. On the other hand, our data showing in Fig. S8 demonstrates that the UPR signaling pathway is dispensable for the ERAC formation of Pma1-2308-mCherry. Meanwhile, cytotoxicity of Pma1-2308-mCherry was enhanced by the *IRE1*-gene deletion (Fig. 3B). It is widely accepted that, in general, the *IRE1*-gene deletion aggravates cellular damage caused by ER-stressing stimuli (Umebayashi *et al.*, 1997; Kubota *et al.*, 2006; Miyagawa *et al.*, 2014; Le *et al.*, 2016).

Intriguingly, the UPR induced by Pma1-2308-mCherry was aggravated by 4-PBA (Fig. 5D). Moreover, viability of cells expressing Pma1-2308-mCherry was decreased by 4-PBA (Fig. 5E). Since, as described above, 4-PBA disperse the ERACs, we assume that these findings represent a beneficial aspect of the ERAC formation. Meanwhile, we cannot exclude a possibility that 4-PBA enhances the Pma1-2308-induced ER stress and its resulting cytotoxicity by another reason. In other words, 4-PBA may be potentially ER-stressing, and may cause a cellular damage which additively or synergistically induces ER stress and harms cells together with cellular expression of Pma1-2308.

Nevertheless, it should also be noted, at least in the case of other ER-stressing stimuli, including cellular treatment with tunicamycin, the UPR was attenuated by 4-PBA (Fig. S9; Kubota *et al.*, 2006; Le *et al.*, 2016). Moreover, according to Kubota *et al.* (2006), 4-PBA supports cellular growth of *ire1Δ* cells in the presence of tunicamycin. These observations demonstrate an effect of 4-PBA to mitigate ER stress, and thus argue against the aforementioned idea that 4-PBA *per se* is potentially ER stressing.

In conclusion, in this study, we disclose some physiological properties of yeast ERACs. We propose that the ERACs are formed through aggregation of aberrant multimembrane-spanning proteins, and work as sequestration sites of multiple client proteins, which are harmful for cells when diffusively distributed across the ER. According to Kakoi *et al.* (2013), multiple cellular proteins, namely COPII-forming factors and cytosolic DnaJ-family proteins, are likely to contribute to the ERAC formation of CFTR. In agreement with our argument presented here, this insight suggests that yeast cells actively form the ERACs when aberrant multimembrane-spanning proteins are accumulated.

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