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The stress granule protein Vgl1 and poly(A)-binding protein Pab1 are required for doxorubicin resistance in the fission yeast *Schizosaccharomyces pombe*

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ABSTRACT

Doxorubicin is an anthracycline antibiotic widely used for chemotherapy. Although doxorubicin is effective in the treatment of several cancers, including solid tumors and leukemias, the basis of its mechanism of action is not completely understood. Here, we describe the effects of doxorubicin and its relationship with stress granules formation in the fission yeast, *Schizosaccharomyces pombe*. We show that disruption of genes encoding the components of stress granules, including *vgl1*$^+$, which encodes a multi-KH type RNA-binding protein, and *pab1*$^+$, which encodes a poly(A)-binding protein, resulted in greater sensitivity to doxorubicin than seen in wild-type cells. Disruption of the *vgl1*$^+$ and *pab1*$^+$ genes did not confer sensitivity to other anti-cancer drugs such as cisplatin, 5-fluorouracil, and paclitaxel. We also showed that doxorubicin treatment promoted stress granule formation when combined with heat shock. Notably, doxorubicin treatment did not induce hyperphosphorylation of eIF2α, suggesting that doxorubicin is involved in stress granule assembly independent of eIF2α phosphorylation. Our results demonstrate the usefulness of fission yeast for elucidating the molecular targets of doxorubicin toxicity and suggest a novel drug-resistance mechanism involving stress granule assembly.

1. Introduction

Although anthracycline antibiotics such as doxorubicin (DXR) are widely used antitumor agents, a major limitation of their use is the occurrence of severe adverse effects, including the development of cardiomyopathy at high cumulative doses [20]. In addition, DXR exerts multiple biological effects, such as the inhibition of topoisomerase II, production of single strand DNA breakage, and membrane damage [15]. Therefore, the exact molecular basis for the antitumor effectiveness of DXR and the mechanism of drug resistance remain elusive [12].

We have been using the fission yeast *Schizosaccharomyces pombe* as a model system for studying the mechanism of drug sensitivity and its relationship with signaling pathway, because of the evolutionary conservation of genes targeted by drugs as well as of signal transduction pathways. Stress granules (SGs) are non-membranous cytoplasmic foci, composed of non-translating messenger ribonucleoproteins (mRNPs) that rapidly accumulate in cells exposed to a broad range of environmental stresses, including oxidative, genotoxic, hyperosmotic, or heat shock (HS) stresses [6,7,18]. In a recent study by Arimoto et al. reported that type 2 stress, including treatment with chemotherapeutic drugs induced SGs formation in higher eukaryotes [2]. SGs have been observed in yeast, such as fission yeast and budding yeast, protozoa, and metazoa [6,7,18]. In budding yeast, the components and kinetics of SG assembly have been extensively studied, and although many components of SGs are highly conserved in this organism, stress-granule assembly and composition can vary in a stress-specific manner [5]. Recently, some of the proteins that localize to SGs in fission yeast have been identified, including the poly(A)-binding protein (Pab1) and Vgl1, a multi-KH-type RNA-binding protein [19]. Additionally, the role of PKA in the regulation of SGs has also been reported [14]. However, the role of SGs in drug resistance in this organism has not been reported.

In this study, we have characterized the role of the components of SGs in DXR tolerance and show that deletion of poly(A)-binding protein (Pab1) and Vgl1 enhance DXR sensitivity in fission yeast. We also demonstrated that DXR treatment specifically promotes SG formation when combined with heat shock.

2. Materials and methods

2.1. Strains, media, and genetic and molecular biology methods

S. pombe strains used in this study are listed in Table 1. The complete medium (yeast extract–peptone–dextrose; YPD), (yeast

Abbreviations: SGs, stress granules; DXR, Doxorubicin; EMM, Edinburgh minimal medium; YES, yeast extract with supplements; GFP, green fluorescent protein; YFP, yellow fluorescent protein; ORF, open reading frame; GST, glutathione-*S*-transferase.

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Table 1
Schizosaccharomyces pombe strains used in this study.

Strain	Genotype	Reference
HM123	h⁻ leu1-32	Our stock
HM528	h⁺ his2	Our stock
KP928	h⁺ his2 leu1-32 ura4-D18	Our stock
SP1365	h⁻ leu1-32 pab1::KanMX4	Our stock
SP1195	h⁻ leu1-32 vgl1::KanMX4	Our stock
SP1422	h^{90} ade6-216 leu1-32 lys1-131 ura4-D18 vgl1::vgl1-GFP-HA-KanMX6	[19]
SP1456	h⁻ pab1-GFP::KanMX6	This study
SP1466	h⁻ leu1-32 nmt1-dcp2-YFP-FLAG-6His::leu1⁺	This study

extract with supplements; YES) and the minimal medium (Edinburgh minimal medium; EMM) have been described previously [13,17]. Standard genetic and recombinant DNA methods [13] were used except where otherwise noted. PCR-based genomic epitope tagging was performed using standard methods [3]. In all cases, proteins were C-terminally tagged with GFP or YFP and expressed at the respective endogenous loci.

2.2. Chemicals

Cisplatin (cis-diamminedichloro-platinum; CDDP), 5-fluorouracil (5-FU), and paclitaxel (PTX) were purchased from Wako (Osaka, Japan). DXR was a kind gift from the Kyowa Hakko Bio Company Ltd. (Tokyo, Japan). Yeast growth media containing each of these chemicals were prepared by mixing stock solutions of these chemicals with the YES medium to achieve the desired drug concentration. For agar media, the stock solution of the appropriate drug was added after autoclaving and cooling of the media to approximately 50 °C. For cisplatin, the stock solution was prepared using DMSO, and DMSO was added to control media at concentrations equivalent to that in the media supplemented with CDDP (see Fig. 1C).

2.3. Protein expression

For protein expression in yeast, the thiamine-repressible *nmt1* promoter was used [11]. Expression was repressed by the addition of 4.0 μg/ml thiamine to EMM. The GFP- or the YFP-fused gene was subcloned into the pREP1, or pREP2-based vectors.

2.4. Growth conditions and stress treatment

Unless otherwise stated, cells were cultivated at 27 °C in EMM or YES rich medium [13]. Before stress treatment, the cells were grown to mid-log phase ($OD_{660\ nm}$ = 0.6). Heat shock was imposed by transferring the culture tubes to a water bath at 42 °C for various times, as indicated in the figures. After heat shock, the culture medium was chilled in ice water for 5 min. The cells were harvested by brief centrifugation at 4 °C.

2.5. Microscopy and miscellaneous methods

Light microscopy methods, such as differential interference contrast and fluorescence microscopy, were performed as previously described [10]. Cell extract preparation and immunoblot analysis were performed as previously described [16]. Data from at least three individual experiments with a minimum of 50 counted cells were used for quantification.

3. Results and discussion

3.1. Effect of doxorubicin on S. pombe cell growth

In order to investigate the relationship between stress granule (SG) formation and DXR sensitivity, we first examined the sensitivity of cells lacking the poly(A)-binding protein (Δ*pab1*), which is a marker protein for SGs and compared the sensitivity of these cells with that of the wild-type cells. We found that Δ*pab1* cells showed

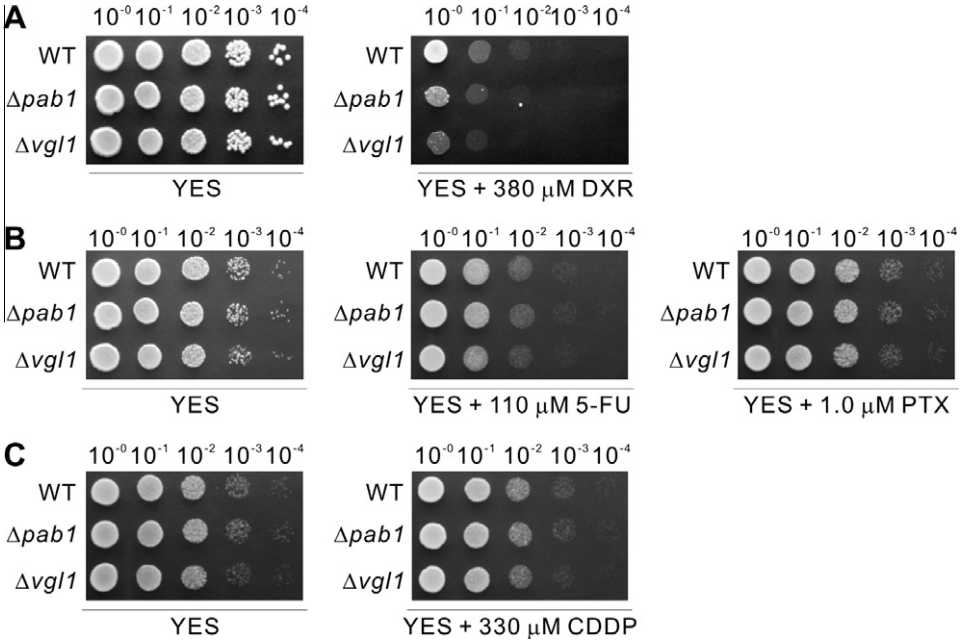

Fig. 1. Effects of DXR on *Schizosaccharomyces pombe* cell growth. (A) Deletion of the poly(A)-binding protein Pab1 and KH-type RNA-binding protein Vgl1 conferred an enhanced sensitivity to DXR (DXR). Serial dilution assay of the wild-type strain (WT) and Δ*pab1* and Δ*vgl1* mutants grown in rich YES medium or YES medium containing the indicated concentrations of DXR. Growth was scored after 3 days of incubation at 27 °C. (B) Deletion of Pab1 and Vgl1 did not cause altered sensitivity to 5-fluorouracil (5-FU) and paclitaxel (PTX). Serial dilution assay of the WT and Δ*pab1* and Δ*vgl1* mutants grown in rich YES medium containing the indicated concentrations of 5-FU and PTX. (C) Deletion of Pab1 and Vgl1 did not cause altered sensitivity to cisplatin (CDDP). Serial dilution assay of the WT and Δ*pab1* and Δ*vgl1* mutants grown in rich YES medium containing the indicated concentrations of CDDP.

greater sensitivity to DXR than did wild-type cells (Fig. 1A; YES + 380 μM DXR). We further examined the sensitivity of cells lacking Vigilin, a multi-KH-type RNA-binding protein, which has recently been identified as a component of SGs [19]. Notably, Δ*vgl1* cells also showed greater sensitivity to DXR (Fig. 1A; YES + 380 μM DXR). Both mutants grew well in YES media, indicating that the growth defect in DXR-containing plates is a result of the toxicity of the drug (Fig. 1A; YES). While this range of concentration is higher than that required for inhibition of tumor development by DXR in cultured mammalian cells [9], we sought to take into account the possibility that the cellular penetrance of DXR might be limited by the *S. pombe* cell wall as well as the possibility that the toxin might be more rapidly metabolized in *S. pombe* cells than in animal cells.

3.2. Analysis of the sensitivity of the SG deletion strains to other cytotoxic treatments

To determine if the DXR-sensitive phenotype associated with Δ*vgl1* and Δ*pab1* cells may be the consequence of general drug sensitivity or specific to DXR, we tested their response to different chemotherapeutic drugs. We examined the ability of the cells to grow in 5-FU (110 μM), PTX (1.0 μM), and CDDP (330 μM). Serial dilutions of stationary cell cultures were spotted on YES plates containing different drugs at concentrations at which the wild-type cells grew well. The growth of both Δ*vgl1* and Δ*pab1* cells was not discernible on treatment with these compounds as compared with the wild-type cells (Fig. 1B and C), indicating that these strains with deletions of SG components specifically displayed an enhanced sensitivity to DXR.

3.3. Doxorubicin promoted the formation of SGs when combined with heat shock

The above findings prompted us to investigate the relationship between DXR resistance and SG formation. In mammalian cells and in both budding and fission yeasts, Pab1 is an SG marker and localizes to these dot-like structures in response to various stresses including heat shock and oxidative stresses [4,8,14]. Therefore, we used GFP-tagged Pab1, the major poly(A)-binding protein of *S. pombe*, and GFP-tagged Vgl1, another SG protein to examine if their localization is affected by DXR treatment. In untreated cells, Pab1-GFP localized to the cytosol (Fig. 2A, panel a). As shown in Fig. 2A, DXR treatment (DXR(+); Fig. 2A, panel b) did not induce the formation of Pab1-positive granules, and heat shock (42 °C, 10 min; Fig. 2A, panel c) slightly increased the formation of Pab1-positive granules. Notably, however, the exposure of the cells to the combination of heat shock and DXR treatment markedly increased the frequency of the Pab1-positive granules (Fig. 2A, panel d, 42 °C 10 min, DXR(+)). We also counted the number of the cells harboring SGs under various treatment conditions to quantitatively assess the effects of heat shock and DXR treatment on SG formation (Fig. 2B). The results showed that DXR(+) treatment did not induce the formation of Pab1-positive granules, and short exposure of the cells to heat shock (HS 7 min) caused a slight increase in the number of cells harboring Pab1-positive granules (Fig. 2B; 0% and 3.9% ± 5.5%, respectively). In contrast, simultaneous treatment of the cells with a short exposure to heat shock and DXR dramatically induced SG formation, as 100% of the cells showed SGs on exposure to heat shock in the presence of DXR (Fig. 2B, DXR+ HS 7 min). A similar additive effect of DXR treatment and HS on SG assembly was also observed when the cells were exposed to heat shock for 10 min (Fig. 2B, HS 10 min), since compared to heat shock alone (44.6% ± 9.62%), DXR treatment increased the frequency of the cells harboring SG (DXR+ HS 10 min; 99.4% ± 0.92%). All cells harbored Pab1-positive granules on heat shock exposure for 20 min in the absence and presence of DXR (Fig. 2A, panels e and f, and Fig. 2B, HS 20 min).

We also examined the stimulative effect of DXR on heat shock-induced SG formation by examining the localization of Vgl1, another component of SGs. Vgl1-GFP was localized to the cytosol in

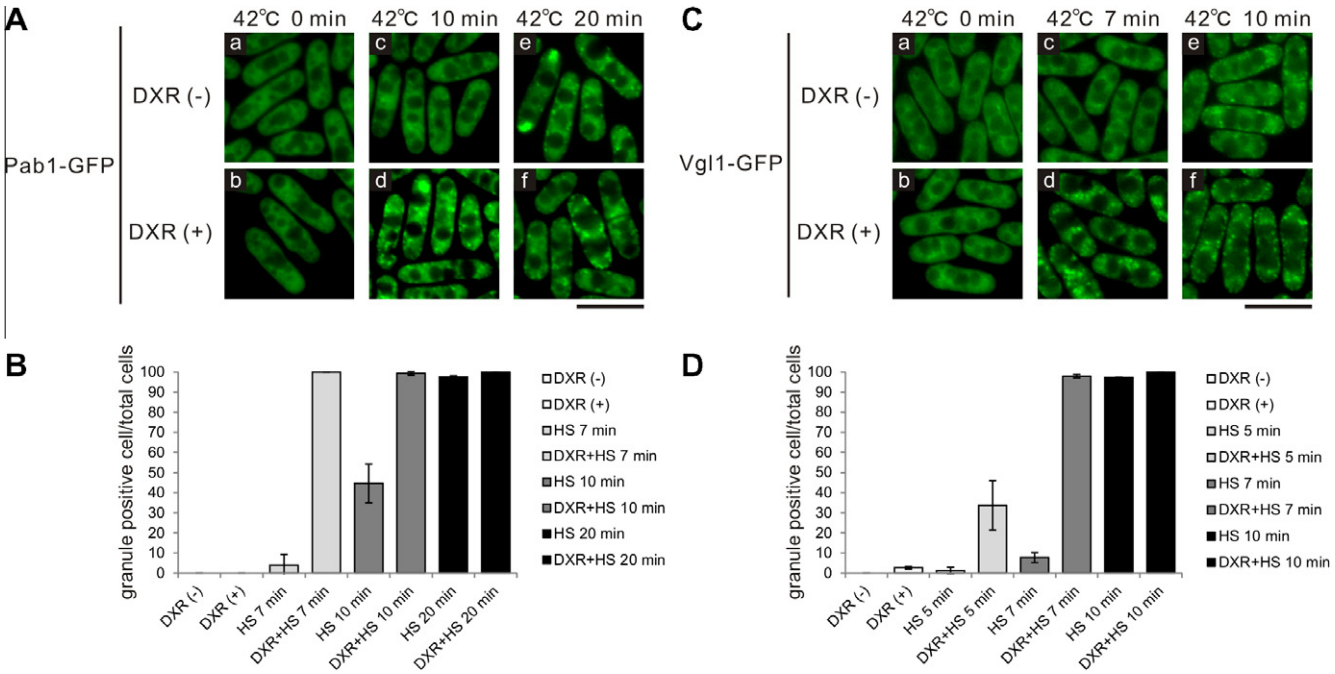

Fig. 2. DXR promotes the formation of stress granules when combined with heat shock. (A) *S. pombe* cells expressing endogenous Pab1-GFP were grown to mid-log-phase in normal YES at 27 °C (42 °C, 0 min) and were incubated for 10-min at 42 °C (42 °C, 10 min) or for 20-min at 42 °C (42 °C, 20 min) in the absence (–) or presence (+) of 380 μM DXR (DXR). Bar, 10 μM. (B) Quantification of cells harboring Pab1-positive granules under various treatment conditions as indicated. (C) *S. pombe* cells expressing endogenous Vgl1-GFP were grown to mid-log-phase in normal YES at 27 °C and were incubated for 7-min at 42 °C (42 °C, 7 min) or for 10-min at 42 °C (42 °C 10 min) in the absence (–) or presence (+) of 380 μM DXR. Bar, 10 μM. (D) Quantification of cells harboring Vgl1-positive granules under various treatment conditions as indicated.

untreated cells (Fig. 2C, panel a). As shown in Fig. 2C, similar to the results obtained with Pab1, Vgl1-positive granules were only slightly induced on either DXR treatment (DXR(+) panel b), or short exposure of heat shock alone (42 °C 7 min panel c). In contrast, simultaneous treatment of the cells with heat shock and DXR clearly resulted in the accumulation of Vgl1-positive granules (Fig. 2C, panel d).

Quantitative analysis also showed that DXR treatment clearly exerted a stimulative effect on heat shock-induced SG assembly (Fig. 2D); a 7 min heat shock exposure in the absence of DXR induced the appearance of Vgl1-positive granules only up to 7.8% ± 2.47% of the cells, whereas the same treatment in the presence of DXR dramatically induced SGs formation up to 98.0% ± 0.92% of the cells (Fig. 2D; DXR+ HS 7 min). Vgl1 localized to SGs upon heat shock for 10 min in the absence and presence of DXR (Fig. 2D; DXR+ HS 10 min; 100%).

3.4. Doxorubicin is involved in SG assembly independent of eIF2α phosphorylation

SG assembly usually requires the stress-induced phosphorylation of the translation initiation factor eIF2α [1]. An allele expressing a phosphomimetic form of eIF2α is sufficient to induce SGs, whereas the expression of a mutant, unphosphorylatable form of eIF2α blocks SG formation upon stress [8]. In fission yeast, the kinetics of appearance of RNA granules after hyperosmotic stress were slower in eIF2α-S52A cells than in wile-type cells [14]. To characterize the effect of DXR in SG formation and its relationship with eIF2α, we examined the phosphorylation levels of eIF2α using anti-phospho eIF2αS^{52} on DXR treatment and heat shock. As shown in Fig. 3, phosphorylation of eIF2α was significantly induced on heat shock in the absence of DXR (Fig. 3 and 36 °C, 3 and 5 min), whereas DXR treatment alone did not increase the phosphorylation of eIF2α (36 °C 0 min, DXR+). In addition, we observed no stimulatory effect of DXR on the phosphorylation of eIF2α induced by heat shock. Thus, DXR is involved in SG assembly independent of eIF2α phosphorylation.

In summary, we found that disruption of the components of SGs in fission yeast resulted in enhanced sensitivity to DXR. Importantly, we have shown that the combination of DXR treatment with a short exposure of heat shock strongly induced the accumulation of two marker proteins of SGs in *S. pombe*, whereas neither treatment produced this effect when administered singly. We also examined the effect of DXR on the formation of P-bodies, which are also RNA granules involved in RNA metabolism/degradation by utilizing the Dcp2 protein, a well-known marker for P-bodies both in fission yeast and in higher eukaryotes. However, in contrast to the results obtained with the SG proteins, Pab1 and Vgl1, the frequency of appearance of the cells harboring Dcp2-YFP-positive granules was not further increased on either DXR or HS treatment (data not shown), indicating that the observed stimulatory effect of DXR on RNA granule formation was specific to SG assembly.

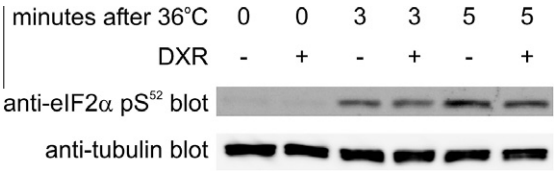

Fig. 3. Effects of DXR on eIF2α phosphorylation. Wild-type cells were grown in YES at 27 °C and treated with heat shock (36 °C) for 0, 3, or 5 min in the absence (−) or presence (+) of 380 μM DXR. Proteins were analyzed by SDS–PAGE and immunoblotting using anti-phospho eIF2α (anti-eIF2α pS^{52} blot), and anti-tubulin (anti-tubulin blot) antibodies.

Our findings that SGs contribute to DXR resistance in *S. pombe* and that DXR facilitated SG formation are quite interesting, given that a previous study in higher eukaryotes suggest the presence of a functional link between SG formation and type 2 stress, including chemotoxicity such as that of etoposide [2]. Therefore, fission yeast SG components are a good model to identify genes associated with DXR toxicity, and our data may prove useful to develop strategies to sensitize tumor cells to DXR by clarifying the role of SGs in the mechanism of action of the drug.

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