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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

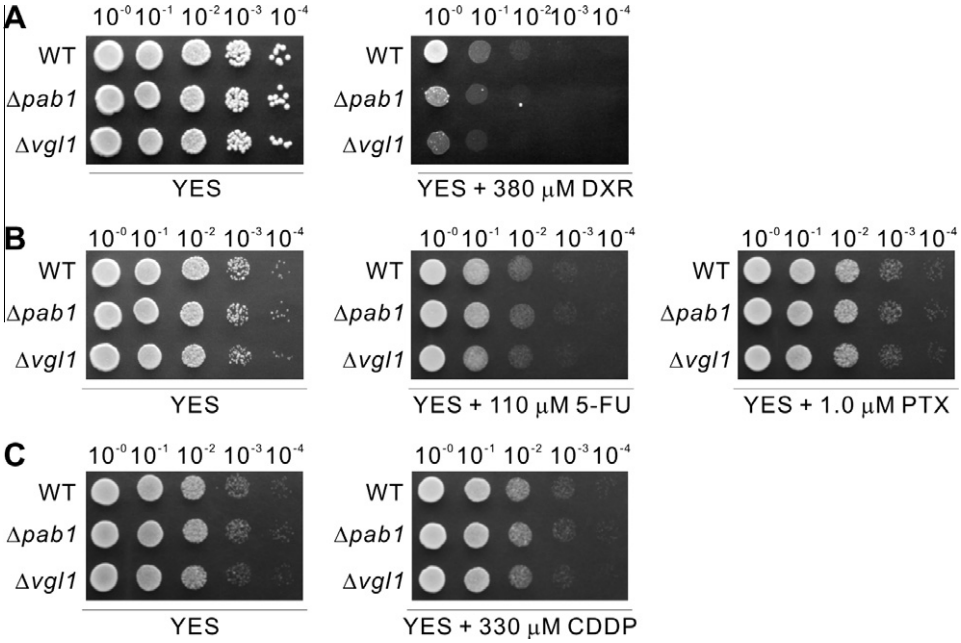

Fig. 1. Effects of DXR on *Schizosaccharomyces pombe* cell growth. (A) Deletion of the poly(A)-binding protein Pab1 and KH-type RNA-binding protein Vgl1 conferred an enhanced sensitivity to DXR (DXR). Serial dilution assay of the wild-type strain (WT) and Δ*pab1* and Δ*vgl1* mutants grown in rich YES 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.