PY motifs of Rod1 are required for binding to Rsp5 and for drug resistance

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Abstract In *Saccharomyces cerevisiae*, the overexpression of *ROD1* confers resistance to *o*-dinitrobenzene (*o*-DNB), a representative of target drugs of glutathione *S*-transferase. The roles of Rod1 in drug resistance have remained to be determined. We isolated the *rog3* mutation as a suppressor mutation of the temperature sensitivity of the strain, in that two of the total four glycogen synthase kinase 3 homologs were deleted. Rog3 is homologous to Rod1, and its overexpression also conferred resistance to *o*-DNB. Furthermore, these two proteins have PY-motifs, and bound to Rsp5, a hect-type ubiquitin ligase. The *rsp5-101* mutant showed sensitivity to *o*-DNB as did the *rod1* mutant, a mutant Rod1 containing altered PY motifs was defective in ability to bind to Rsp5 and in conferring *o*-DNB resistance. These results suggest that interaction of Rod1 and Rsp5 is important for drug resistance.

Key words: Drug resistance; Glutathione *S*-transferase; Ubiquitin ligase; *o*-dinitrobenzene; GSK-3; *Saccharomyces cerevisiae*

1. Introduction

Treatment of humans with tumors can be hampered by the resistance of cancer cells to a broad range of cytotoxic drugs. Using the yeast *Saccharomyces cerevisiae*, an excellent model for studying mammalian multi-drug resistance, a large number of genes mediating the resistance to toxic drugs has been identified. Proteins encoded by these genes are divided generally into two main groups. One consisting of membrane ATP-binding cassette (ABC) transporter proteins such as Pdr5, Snq2, Ycf1, and Yor1, and the other regulatory factors such as Pdr1, Pdr3, Pdr13, Ngg1, Yrr1, yAP1, and Cmk1, which control these transporters at transcriptional or the post-translational levels (reviewed in [1]).

In eukaryotic cells, a large number of lipophilic toxins is conjugated with glutathione, often by the glutathione *S*-transferase (GST) enzyme. Toxic drugs covalently conjugated with glutathione are recognized and eliminated from the cytosol by the GS-X (glutathione *S*-conjugate transporting ATPase) pump. In *S. cerevisiae*, Ycf1, a vacuolar membrane-bound GS-X pump, transports GS-conjugates from the cytosol to the vacuole. The *ycf1* disruptant shows higher sensitivity to 1-chloro-2,4-dinitrobenzene (CDNB), one of the substrates of the GST enzyme [2]. In research done on the detoxification pathway involving the glutathione-conjugating system, *ROD1* was found to confer resistance to *o*-dinitrobenzene (*o*-DNB), another substrate of the GST enzyme [3]. The *ROD1*-overexpressed cell shows resistance to *o*-DNB, and the *rod1* disruptant shows sensitivity to *o*-DNB. Rod1 is co-fractionated at the plasma membrane [3], however, as deduced by its primary amino acid sequence, it is not likely that Rod1 is a member of the ABC transporter proteins. Whether Rod1 regulates transporters has not been determined nor have its functions been elucidated [3].

Rsp5 is an essential hect-type ubiquitin ligase and plays roles in several cellular functions involving protein degradation and transcriptional regulation [4–6]. Rsp5 has three WW domains. The WW domain is known to bind to the PY motif [7], and the PY-motif-containing proteins Bul1 and Bul2 are reported to bind to Rsp5 and may promote functions of Rsp5 in protein degradation [5,8]. However, factors other than Bul1 and Bul2 have never been found to regulate Rsp5 functions.

S. cerevisiae has four genes, *MCK1*, *MDS1*/*RIM11*, *MRK1*, and *YOL128c*, which encode homologs of mammalian GSK-3. GSK-3s of *S. cerevisiae* act in the transcriptional regulation of meiotic genes [9–11], the chromosomal segregation processes at mitosis [12], and the cell cycle delay by the addition of Ca^{2+} [13]. In an earlier study, we identified five exogenous mutations, *rog1–rog5*, as suppressor mutations of the temperature sensitivity of the *mck1 mds1* mutant. Rog1 was degraded dependently on Rsp5 and GSK-3 [5], indicating that Rsp5 functionally interacts with GSK-3. Rog3, isolated from another suppressor mutation of the *mck1 mds1* mutant, is homologous with Rod1.

We now report on Rog3 and its homolog Rod1. Both Rog3 and Rod1 have two PY motifs and interact with Rsp5. Further, a mutant Rod1, which decreases its binding ability to Rsp5, does not confer resistance to *o*-DNB. Our finding suggests the involvement of Rsp5 and the importance of the interaction between Rsp5 and Rod1 in drug resistance.

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Abbreviations: *o*-DNB, *o*-dinitrobenzene; GST, glutathione *S*-transferase; ABC transporter, ATP binding cassette transport protein; CDNB, 1-chloro-2,4-dinitrobenzene

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2. Materials and methods

2.1. Materials and chemicals

pTS010, pTS011, pTS012 [14], and functional *RSP5* plasmids, pHY22 (myc-Rsp5) and YCp-HA-RSP5, were kindly provided by Dr. Y. Kikuchi (University of Tokyo). GST fusion proteins and maltose-binding protein (MBP) fusion proteins were purified from *Escherichia coli* according to the manufacturer's instructions. Other materials and chemicals were obtained from commercial sources.

2.2. Strains and genetic manipulations

S. cerevisiae strains used in this study were KA31a (wild type), YTA002K (*mck1::TRP1 mds1::HIS3*), YAT2-1C (*rsp5-101*), W303a (wild type) [5], YTA021K (*MAT*a *his3 leu2 ura3 trp1 mck1::TRP1 mds1::HIS3 rog3::LEU2*), and YTA122K (*MAT*a *his3 leu2 ura3 trp1 rod1*). Media and methods for mating, sporulation, tetrad analysis, and transformation were as described [15].

2.3. Plasmid constructions

To construct pTA054 (pKT10-HA-Rog3), the hemagglutinin (HA)-tagged *Eco*RI–*Sal*I PCR fragment of *ROG3* was inserted between the *Eco*RI and *Sal*I sites of pKT10 [5]. pTA055 (pKT10-HA-Rog3^{QA}) was prepared from pTA054 by replacing sequences 5′-CCCCCA-3′ (nucleotides +1378 to +1383) and 5′-CCTCCT-3′ (nucleotides +1873 to +1878) with 5′-CAGGCC-3′. pTA061 (YEplac195-Rod1–HA) and pTA062 (YEplac112-Rod1–HA) were constructed by inserting the *Sal*I–*Sma*I PCR fragment of nucleotides −771 to +2510 of *ROD1* into the *Sal*I and *Sma*I sites of pTS011 and pTS012 [14], respectively. pTA063 (YEplac195-Rod1^{QA}–HA) and pTA064 (YEplac112-Rod1^{QA}–HA) were prepared from pTA061 and pTA062, respectively, by replacing sequences 5′-ACCTCCC-3′ (nucleotides +1458 to +1464) and 5′-TCCACCGGC-3′ (nucleotides +1965 to +1973) with 5′-ACAGGCC-3′ and 5′-TCAGGCCGC-3′, respectively.

In pTA057 (*rog3::LEU2*), nucleotides +52 to +2035 of *ROG3* were replaced by *LEU2*. To create pTA066 (*rod1::URA3*), nucleotides +1 to +2551 of *ROD1* were replaced by the 2.4-kb *Hin*dIII fragment of TAp700 [5]. For expression of fusion proteins in *E. coli*, pTA063 was constructed by inserting the 0.7-kb *Xba*I fragment of *ROG3* into the multi-cloning site of pGEX-KG.

2.4. Disruption of ROG3 and ROD1

Cells were transformed by pTA057 cut with *Dra*I or by pTA066 cut with *Sac*I and *Sal*I, and disruption of the *ROG3* gene of Leu$^+$ transformants or disruption of the *ROD1* gene of Ura$^+$ transformants was confirmed by Southern blotting analysis. For pop-out of the *URA3* gene, *rod1::URA3* cells were incubated on a synthetic complete medium plate containing 0.1% 5-fluoro-orotic acid and the colonies formed were obtained.

2.5. Immunoblot analysis and immunoprecipitation

Exponentially growing yeast cells in 25 ml medium were collected by centrifugation, washed once with lysis buffer (50 mM Tris–HCl, pH 7.5, 0.3 M mannitol, 0.1 M KCl, 1 mM EGTA) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml antipain, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A [16], and resuspended in 100 μl of ice-cold lysis buffer. The cells were broken by vortex mixer with glass beads for 5 min at 4°C. Then, 100 μl of lysis buffer was added, the homogenate was mixed vigorously, then was centrifuged at 14 000×*g* for 5 min. The supernatant was recovered and centrifuged at 14 000×*g* for 5 min, and the resultant supernatant fraction served as cell lysates which were probed with anti-HA and anti-myc antibodies [17]. For the immunoprecipitation assay, the lysates were incubated with the anti-HA antibody and protein A–Sepharose, then precipitated by centrifugation. The immunoprecipitates were washed five times with the lysis buffer.

2.6. Other procedures

Nucleotide sequences were determined using Thermo Sequenase pre-mixed cycle sequencing kits (Amersham Pharmacia Biotech, Uppsala, Sweden). Southern hybridization was done as described [18].

3. Results

3.1. Rog3 has PY motifs

In foregoing work, we obtained five insertional mutations (named *rog1–rog5*) that suppressed temperature sensitivity of the *mck1 mds1* double null mutant [5]. Among the five, suppression by the *rog3* mutation was weak. Sequence analysis revealed that the *LEU2* fragment is inserted just after nucleotide +302 of the *YFR022W* in the *rog3* mutant. To confirm that the suppression was caused by a loss of function of *ROG3*, the open reading frame of *ROG3* was deleted. Disruption of *ROG3* also slightly suppressed temperature sensitivity of the *mck1 mds1* double null mutant (Fig. 1), as did the originally isolated *rog3* mutation. In screening and confirmation steps for the suppression, we used the *mck1 mds1* double null mutant instead of the disruptant of all four GSK-3 genes, since the double null mutant showed the same phenotypes as the quadruple null mutant and was easier to use for genetic analysis. Despite weakness of its suppression phenotype, we continued to examine the *rog3* mutant since *ROG3* (*YFR022W*) encodes a protein with two PY motifs (Fig. 2A).

3.2. Rog3 binds to Rsp5

The PY motif of Bul1 was reported to be important for in vivo complex formation with Rsp5 [19]. To determine if PY motifs of Rog3 bind to Rsp5, the GST-tagged fragment, including the PY motifs of Rog3 (GST-Rog3PY-(431–679)), was purified from *E. coli* and incubated with MBP–Rsp5 or MBP [5] immobilized to amylose resin. GST-Rog3PY-(431–679) co-precipitated with MBP–Rsp5 but not with MBP (Fig. 2Ba), thereby suggesting that the PY motifs of Rog3 directly bind to Rsp5. To examine an interaction of Rsp5 and Rog3, cell lysates of wild-type strains expressing myc-Rsp5 (pHY22) or HA–Rog3 (pKT10-HA-Rog3) were mixed, then immunoprecipitated with the anti-HA antibody. myc-Rsp5 was detected in the HA–Rog3 immune complex (Fig. 2Bb), which suggested that Rog3 forms a complex with Rsp5.

3.3. Rog3 is a homolog of Rod1

Rog3 shares 43% identity with Rod1. Since overexpression

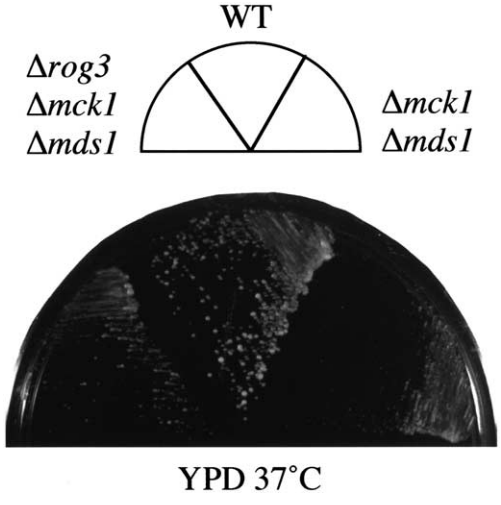

Fig. 1. Deletion of *ROG3* weakly suppressed temperature sensitivity of the Δ*mck1* Δ*mds1* null mutant. Cells of KA31a (WT), YTA002K (Δ*mck1* Δ*mds1*), and YTA021K (Δ*rog3* Δ*mck1* Δ*mds1*) were incubated on a YPD plate for 2 days at 37°C.

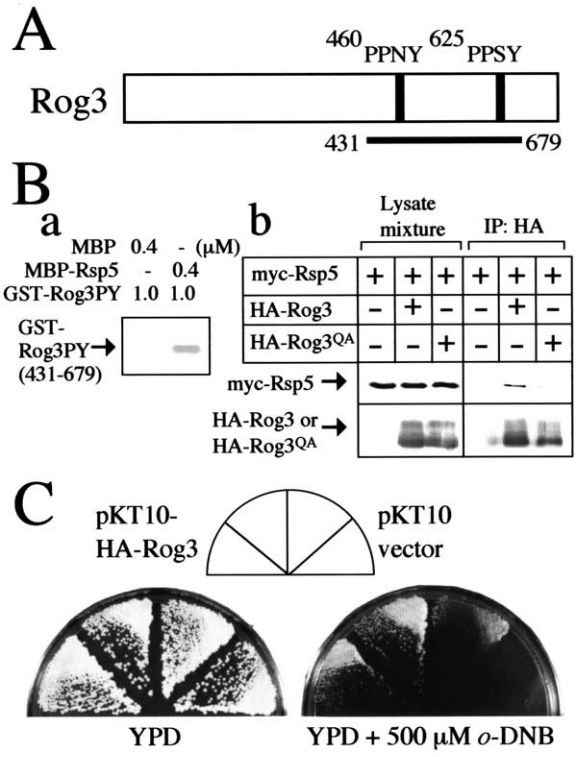

Fig. 2. Rog3 bound to Rsp5. A: Schematic representation of Rog3 is shown. The PY motifs of Rog3 and the region fused to GST in GST-Rog3PY-(431–679) are shown by black boxes and the solid bars, respectively. B: Binding assay between Rog3 and Rsp5 was done. The GST-Rog3PY-(431–679) purified from *E. coli* was incubated with MBP–Rsp5 or MBP immobilized to amylose resin at 4°C for 1 h, and the precipitates were probed using an anti-GST antibody (a). Lysates of cells expressing myc-Rsp5 (pHY22), HA–Rog3 (pKT10-HA-Rog3), or HA–Rog3^{QA} (pKT10-HA-Rog3^{QA}) were mixed and immunoprecipitated with the anti-HA antibody. Lysates and the precipitates were probed with an anti-myc and anti-HA antibodies (b). C: Overexpression of Rog3 conferred *o*-DNB resistance. KA31a cells with pKT10 vector or pKT10-HA-Rog3 were incubated on a YPD plate with or without 500 μM *o*-DNB at 26°C for 4 days.

of *ROD1* was reported to confer resistance to *o*-DNB [3], we asked if *ROG3* would show a similar phenotype. Overexpression of *ROG3* by pKT10-HA-Rog3, in which HA-tagged Rog3 was expressed from *TDH3* promoter, conferred resistance to *o*-DNB (Fig. 2C). Therefore, we considered that Rog3 and Rod1 are functional homologs, although, unlike the *rod1* mutant, the *rog3* mutant did not show *o*-DNB sensitivity (data not shown). Since Rod1 has two PY motifs (Fig. 3A), possible physical interaction between Rod1 and Rsp5 was examined. Cells co-expressing myc-Rsp5 (pHY22) and Rod1–HA (YEplac112-Rod1–HA) were lysed then immunoprecipitated with the anti-HA antibody. myc-Rsp5 was detected in the Rod1–HA immune complex (Fig. 3B), suggesting that Rod1 forms a complex with Rsp5 in intact cells.

3.4. PY motifs of Rod1 are important for binding to Rsp5 and for resistance to o-DNB

We examined whether Rod1 and Rog3 bind Rsp5 through their PY motifs. Two PY motifs of Rod1, PPNY and PPAY, were mutated to QANY and QAAY, respectively (Rod1^{QA}). The binding of myc-Rsp5 to Rod1^{QA}–HA was significantly reduced as compared with that to wild-type Rod1–HA (Fig. 3B). When the amounts of myc-Rsp5 bound to Rod1–HA or Rod1^{QA}–HA were quantified by NIH Image Software, the binding of myc-Rsp5 to Rod1^{QA} was reduced to less than 28% of that to Rod1. Two PY motifs of Rog3, PPNY and PPSY, were mutated to QANY and QASY, respectively (HA–Rog3^{QA}). We also found the reduction in the binding activity of Rog3^{QA} (Fig. 2Bb). Its binding activity to Rsp5 was reduced to less than 22% of wild-type Rog3. This suggests that the PY motifs of Rod1 and Rog3 are important for binding to Rsp5. Furthermore, expression of Rod1–HA but not Rod1^{QA}–HA rescued the *o*-DNB sensitivity of *rod1* cells (Fig. 3C). This means that Rod1^{QA}–HA is defective as to resistance to *o*-DNB, whereas Rod1–HA is functional. As this finding suggested the involvement of Rsp5 in drug resistance, we asked if Rsp5 is involved in the resistance to *o*-DNB. To this end, we investigated the *o*-DNB sensitivity of the *rsp5-101* mutant that is known to show temperature sensitivity [19]. This mutant was sensitive to *o*-DNB at the

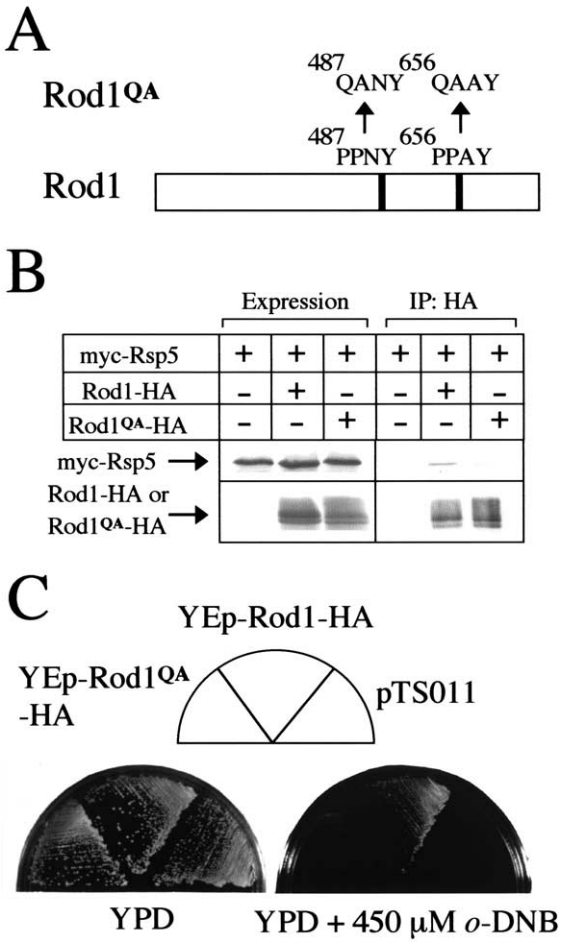

Fig. 3. Rod1 and Rsp5 physically interacted in vivo. A: Schematic representation of Rod1 and Rod1^{QA} is shown. B: myc-Rsp5 was co-immunoprecipitated with Rod1–HA. Cell lysates of W303a cells carrying pTA062 (Rod1–HA), pTA064 (Rod1^{QA}–HA), or pTS012 with pHY22 (myc-Rsp5) were directly probed with an anti-myc or an anti-HA antibody. The immunoprecipitates obtained with the anti-myc antibody were probed using the same antibodies. C: Expression of *ROD1* suppressed *o*-DNB sensitivity of the *rod1* mutant. *rod1* mutant cells (YTA122K) with pTA061 (Rod1–HA), pTA063 (Rod1^{QA}–HA), or pTS011 were incubated on a YPD plate with or without 450 μM *o*-DNB at 26°C for 4 days.

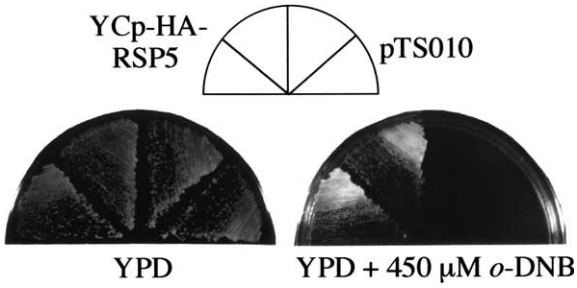

Fig. 4. The *rsp5-101* mutant showed sensitivity to *o*-DNB. The *rsp5-101* (YAT2-1C) cells with YCp-HA–RSP5 or pTS010 were incubated on a YPD plate with or without 450 μM *o*-DNB at 26°C for 3 days.

non-restrictive temperature and expression of HA–Rsp5 in the *rsp5-101* mutant cells rescued the phenotype (Fig. 4). Taken together, these results suggest that Rod1 and Rsp5 play a role in tolerance to *o*-DNB and that the interaction of Rod1 and Rsp5 is important for *o*-DNB resistance.

4. Discussion

The detoxification pathway dependent on glutathione-conjugation plays an important role in resistance to a large number of drugs (reviewed in [20]). Rod1 may be involved in this pathway as its overexpression confers resistance to *o*-DNB [3]. We found that Rog3, a Rod1 homolog, genetically interacted with GSK-3 and that both Rog3 and Rod1 have two PY motifs. Since GSK-3 functionally interacts with Rsp5 [5], a functional interaction between Rog3, Rod1 and Rsp5 was predicted. We found that Rog3 and Rod1 did physically interact with Rsp5. We used lysates of cells overexpressing myc-Rsp5, Rod1–HA, and HA–Rog3 in the co-immunoprecipitation assay. When we used extracts of cells harboring CEN-based plasmids, complex formation of Rsp5 with Rod1 or Rog3 could not be observed (data not shown). The interaction of these proteins may be transient, and therefore difficult to detect in intact cells at the endogenous level. In the co-immunoprecipitation assay, the mutations in the PY motifs of Rod1 and Rog3 caused reduction in their binding ability to Rsp5 (Figs. 2 and 3). Since the overexpression of Rod1 but not $Rod1^{QA}$ conferred resistance not only to *o*-DNB but also to CDNB (data not shown), another target of GS-conjugate, these results suggested an involvement of Rsp5 and Rod1 in the detoxification pathway of GS-conjugate. As Rsp5 plays roles in protein degradation and transcription [4–6], we are now investigating whether or not Rod1 and Rsp5 regulate the ABC transporters involved in drug resistance.

We think that Rog3 and Rod1 are regulators rather than substrates of Rsp5, since the amount of Rod1–HA is usually the same level as that of $Rod1^{QA}$–HA in wild-type cells transformed by each plasmid (data not shown). Bul1 and Bul2, the PY motif-containing proteins binding to Rsp5, may also be regulators of Rsp5 [18]. Other hect-type ubiquitin ligases possessing WW domains may be regulated by PY motif-containing proteins.

The *rog3* mutation was isolated as an exogenous suppressor of the *mck1 mds1* mutant, but the level of suppression was weak. Further, the *rod1* mutant did not suppress temperature sensitivity of the *mck1 mds1* mutant, and the degradation of Rog1, which is regulated by Rsp5 and GSK-3, was not affected by deletion of *ROG3* and *ROD1* genes (data not shown). The mechanism as to how the *rog3* mutation partially suppressed the defect of the *mck1 mds1* mutant was not elucidated. Despite levels of their suppressive potentials to the *mck1 mds1* mutant, the drug resistance may be mainly regulated by *ROD1* rather than *ROG3*, as *rod1* but not *rog3* mutants showed *o*-DNB sensitivity. These differences between *ROD1* and *ROG3* may be partly caused by differences in expression levels of encoding proteins, since our preliminary data indicated that the amount of Rog3 is much lower than that of Rod1. It is also possible that these two genes share some but not all functions.

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