Glutathione-dependent detoxification reactions are catalyzed by the enzyme glutathione S-transferase and are important in drug resistance in organisms ranging from bacteria to humans. The yeast Issatchenka orientalis expresses a glutathione S-transferase (GST) protein that is induced when the GST substrate o-dinitrobenzene (o-DNB) is added to the culture. In this study, we show that overproduction of the I. orientalis GST in Saccharomyces cerevisiae leads to an increase in o-dinitrobenzene resistance in S. cerevisiae cells. To recover genes that influence o-DNB resistance in S. cerevisiae, a high copy plasmid library was screened for loci that elevate o-DNB tolerance. One gene was recovered and designated ROD1 (resistance to o-dinitrobenzene). This locus was found to encode a novel protein with no significant sequence similarity with proteins of known function in the data base. An epitope-tagged version of Rod1p was produced in S. cerevisiae and shown to function properly. Subcellular fractionation experiments indicated that this factor was found in the particulate fraction by differential centrifugation. Overproduction of Rod1p leads to resistance to not only o-DNB but also zinc and calcium. Strains that lack the ROD1 gene are hypersensitive to these same compounds. Rod1p represents a new type of molecule influencing drug tolerance in eukaryotes.

Cancer cells can often acquire a broad range resistance to cytotoxic drugs through alteration of certain genes. The most extensively described of these alterations is a change in expression of an ATP binding cassette transporter protein encoded by the MDR1 gene (1). Increased production of this factor leads to a strong cross-resistant phenotype to a variety of chemotherapeutic agents and is a major problem in clinical treatment of human tumors.

Multidrug or pleiotropic drug resistance in Saccharomyces cerevisiae has served as a useful model for mammalian multidrug resistance. A large number of loci involved in the yeast pleiotropic drug resistance phenotype have now been identified due, in large part, to the ease of genetic manipulation in this organism. The identities of these gene products have been recently reviewed (2) and fall generally into two main classes:

- Membrane transporter proteins and transcriptional regulatory proteins. Specific examples of these general classes of factors include the ATP binding cassette transporter Pdr5p (3–5) and the transcriptional regulatory protein yAP-1 (6). Pdr5p is believed to function as a drug efflux pump (7), while yAP-1 activates the expression of a number of genes that directly act to confer drug resistance (8–10).

- One of the genes activated by yAP-1 is the GSH1 locus which encodes the γ-glutamylcysteine synthetase enzyme (8). This protein catalyzes the rate-limiting step in glutathione biosynthesis (11). Transcriptional control of GSH1 by yAP-1 is essential for normal cadmium tolerance (8). These observations indicate that yAP-1 is important in regulating glutathione production, which in turn is important in detoxification reactions in yeast.

Glutathione has been found to be an important participant in the inactivation of a large number of drugs in eukaryotic cells (12). The covalent attachment of glutathione to target compounds is often catalyzed by the enzyme glutathione S-transferase (GST) (13). The involvement of GSTs in glutathione-dependent detoxification reactions has been well documented in many eukaryotic cells, but no GST has yet been found in S. cerevisiae. A fungal GST-encoding gene has been isolated from the yeast Issatchenka orientalis (14). Expression of the I. orientalis GST locus is inducible by the GST substrate o-dinitrobenzene (o-DNB). Although it is attractive to speculate that production of the I. orientalis GST protein is involved in detoxification of o-DNB, there is no direct evidence to support this belief.

We expressed the I. orientalis GST in S. cerevisiae and found that this factor does elevate resistance to o-DNB. In addition, we screened a high copy plasmid library of S. cerevisiae genomic DNA for loci present in this organism that would elevate o-DNB tolerance. A gene was recovered that was designated ROD1 (resistance to o-dinitrobenzene). This locus conferred resistance to o-DNB, calcium, and zinc when carried on a high copy plasmid. The ROD1 gene product (Rod1p) represents an unusual class of resistance determinant and shares no significant homology with proteins of known function. ROD1 is not essential to the cell, but a Δrod1 mutant strain possesses a hypersensitive phenotype to o-DNB, zinc, calcium, and diamide.

MATERIALS AND METHODS

Yeast Strains and Media—The genotypes of the yeast strains used in this study are listed in Table I. Yeast transformations were performed by the lithium acetate method (15). Standard YPD medium and minimal medium were prepared as described by Sherman (16). Solutions of o-DNB, ZnCl2, diamide, or CaCl2 sterilized by filtration were added to standard yeast media to test resistance phenotypes. Assay for glutathione-dependent detoxification reactions was performed as described by Hallstrom (12).

The abbreviations used are: GST, glutathione S-transferase; o-DNB, o-dinitrobenzene; PCR, polymerase chain reaction; kb, kilobase pair(s).
one S-transferase activity was carried out as described with o-DNB as the substrate (17).

Isolation of the ROD1 Gene—A YEp24-based genomic library (18) was introduced into YE6210 by a high efficiency transformation method (19). Ura− transfectants were selected on minimal medium and tested for o-DNB resistance by replica plating onto YPD medium containing 400 μM o-DNB. Ura− and o-DNB-resistant transfectants were then streaked on minimal medium containing a range of concentrations of o-DNB. A plasmid which conferred high levels of o-DNB resistance was recovered from one of these transfectants. This plasmid, designated 24-1-A, was then re-introduced into YE6210, resulting in o-DNB resistance.

Plasmids—A DNA fragment corresponding to the open reading frame of the l. orientalis GST locus was prepared by PCR. Two primers (GGG GAT CCA AAA TGA CTT TCG CAA CTG TTT A and CCC AAG AAC TGC AGC AGG TCT TCC) were used to amplify a 0.6-kb fragment from l. orientalis genomic DNA. The PCR fragment was cloned as a BamHI-HindIII insert into the yeast expression vector pJAW8 (20). The amplified and cloned fragment was sequenced to ensure that no errors had occurred during the PCR reaction. This 2-μm plasmid, which expressed the l. orientalis GST gene under control of the yeast ADH1 promoter, was designated pSM84. pAW39 and pAW40 were made by replacing the 1.3-kb BamHI fragment and 1.7-kb Xhol fragment, respectively, from the original 24-1-A plasmid, pAW42 and pAW43 were constructed by cloning the 3.3-kb BamHI fragment and the 1.7-kb EcoR1-HindIII fragment of 24-4-1 into the BamHI site and the EcoRV-BamHI II fragment of 2-μm pRS426, respectively. pAW49 was constructed by replacing the 1.3-kb HindIII fragment of pAW43 with the 2.0-kb HindIII fragment of pAW42, such that the partial open reading frame of ROD1 was extended to the BamHI site. pAW47 contained the same insert as pAW49 but was cloned in the opposite orientation. For the purpose of producing the disruption mutant, pAW58 was constructed by inserting a 3.3-kb BamHI fragment of 24-4-1 into the BamHI site of the pBluescript KSII(−) vector. For the phenotypic assay, pSEY18-R2.5, a 2-μm vector containing the wild-type YAP1 gene, was used (20).

Disruption of the ROD1 Gene—Gene disruptions were constructed by the one-step disruption method (21). The ROD1 gene disruption plasmid was constructed by deleting the 1.4-kb BglII fragment present in pAW58 between positions –81 and –120 (relative to the translation start site) of ROD1 and then replacing this segment with a 4.3-kb BglII-BamHI fragment containing hisG-URA3-hisG fragment. The subsequent chimera was designated pAW60 and was digested with BamHI prior to transformation into the wild-type strain YE6210. Appropriate recombinants were confirmed by Southern blotting, and the URA3 gene was cured by treatment with 5-fluoroorotic acid as described previously (23). The rod1-1::hisG strain was designated YAW14. The rod1-1::hisG and yap1-1::hisS double mutant strain (YAW15) was constructed by disruption of the ROD1 gene in the SM10 (Δyap1) (20) background.

Addition of a c-Myc Epitope to ROD1—To place a c-Myc epitope near the C terminus of the ROD1 coding sequence, digoxigenin-labeled primers (5′-CTACAAGCTTCTTCAGGAAAAATGCTGCTG-3′, 5′-GATCCGAAAACCATTTCTTCTGGAGATCAGT-3′) were synthesized containing the 9E10 epitope coding sequences (24) flanked by SacI and BamHI sites. The 335-base pair SacI/BamHI fragment of pAW49 was replaced by the digoxigenin-labeled probe. The resulting plasmid was named pAW75. pAW75 was examined by nuclease digestion sequence analysis to confirm proper orientation of the inserts and preservation of the correct reading frame. Subcellular Fractionation and Immunoblot Analysis—Total cellular protein extracts were prepared as described previously (25). The association of Rod1p with membranes was determined as described by Graham et al. (26) with slight modifications. Cells in the exponential growth phase were mechanically disrupted and centrifuged at 1,000 × g to eliminate cell walls and unbroken cells. The resulting cell crude extract was divided into five equal portions. The first portion was stored at −80°C. The second and third portions were subjected to 10,000 × g and 100,000 × g centrifugation, respectively. The fourth and fifth portions were subjected to 100,000 × g centrifugation after treatment with 0.1 M Na2CO3 (pH 11.0) or 1% Triton X-100. Plasma membrane-enriched protein fractions were prepared as described by Baltz et al. (3).

The different protein fractions obtained were electrophoresed on sodium dodecyl sulfate (SDS)-8% polyacrylamide gels before being transferred to nitrocellulose (27). Monoclonal antibody 9E10 (24) was used as the primary antibody to detect the c-Myc epitope. Monoclonal antibodies against carboxypeptidase Y (Molecular Probes), tubulin (YOL134, Seralabs), and Ras mAb Y13-259 were used as primary antibody for controls. Detection of the primary antibody was performed by using the chemiluminescence detection system (ECL, Amer sham Corp.).

RESULTS

Cloning of a Gene Conferring o-Dinitrobenzene Resistance by DNA Amplification—Our previous work has shown that control of glutathione biosynthesis is important for cadmium resistance in S. cerevisiae (8). Glutathione-dependent detoxification mechanisms in other organisms often require the function of the enzyme GST (12). No GSTs have been isolated from S. cerevisiae, but a GST-encoding gene has been isolated from l. orientalis (14). This l. orientalis GST is induced by the presence of the GST substrate o-dinitrobenzene and is believed to act to detoxify this compound, although this suggestion has not been directly tested (28). We prepared the l. orientalis GST gene by PCR in order to test if this fungal GST could influence o-DNB resistance in S. cerevisiae.

A plasmid (pSM84) expressing the l. orientalis GST gene was able to tolerate 250 μM o-DNB, while the expression vector alone was only able to support growth below 200 μM o-DNB (Fig. 1). While resistance to o-DNB was increased, S. cerevisiae transformants carrying pSM84 did not show increased resistance to several other inhibitors tested, including cycloheximide, cadmium, and 1,10-phenanthroline (data not shown). Furthermore, GST enzyme activity present in S. cerevisiae transformants carrying pSM84 or a high copy YAP1 plasmid was elevated relative to the vector alone. GST activity produced by cells carrying pSM84 was 24.4 milliunits/mg, while cells bearing the expression vector only produced 2.8 milliunits/mg. Transformants carrying a 2-μm YAP1 elevated GST activity to 6.4 milliunits/mg. Also Δyap1 yeast strains were found to be hypersensitive to o-DNB (see below). These data suggested that S. cerevisiae possesses an o-DNB detoxification system that involves YAP1 and might also employ other glutathione-dependent proteins (like GST). To further explore the mechanisms of detoxification of o-DNB, we screened a high copy plasmid library for loci that could elevate resistance to this compound.

The wild-type S. cerevisiae strain SEY6210 was transformed with DNA from a YEp24 S. cerevisiae genomic library (18). Approximately 10,000 Ura+ transformants were tested for o-DNB tolerance by replica plating to YPD plates containing 400 μM o-DNB. Plasmid DNAs from 10 o-DNB resistance colonies were recovered and reintroduced back into the SEY6210 strain. Two different classes of plasmid DNAs were able to reproducibly restore resistance to 400 μM o-DNB. We focused our attention on the function of the plasmid containing the

| Designation | Genotype | Source |
|-------------|----------|--------|
| SEY6210     | MATα, leu2-3,112, ura3-52, his3-1200, trp1-α901, lys2-801, suc2-19, Mal- | Scott Emr |
| SM10        | MATα, leu2-3,112, ura3-52, his3-1200, trp1-α901, lys2-801, suc2-19, Mal-, yap1-1::HIS3 | Ref 20 |
| PB1         | MATα, leu2-3,112, ura3-52, his3-1200, trp1-α901, lys2-801, suc2-19, Mal- | Ref 8 |
| YAW10       | MATα, leu2-3,112, ura3-52, his3-1200, trp1-α901, lys2-801, suc2-19, Mal-, yap1-1::HIS3, cad1-1::hisG | This study |
| YAW14       | MATα, leu2-3,112, ura3-52, his3-1200, trp1-α901, lys2-801, suc2-19, Mal-, rod1-1::hisG | This study |
| YAW15       | MATα, leu2-3,112, ura3-52, his3-1200, trp1-α901, lys2-801, suc2-19, Mal-, yap1-1::HIS3, rod1-1::hisG | This study |
smallest insert of \textit{S. cerevisiae} genomic DNA. This plasmid was designated 24-4-1. Restriction mapping of this plasmid indicated that the insert was approximately 8.0 kb (Fig. 2). The 

Physical Analysis of the ROD1 Gene—To identify the location of ROD1, several different subclones of the \textit{S. cerevisiae} genomic DNA present in 24-4-1 were constructed. The resulting plasmids were introduced into SEY6210. The transformants were tested for resistance in YPD medium containing 400 \( \mu \)M o-DNB (Fig. 2). Transformants bearing either pAW39 or pAW40 were not able to grow on medium containing o-DNB. This indicated that either the BamHI fragment or XhoI fragment contained at least part of the gene responsible for resistance to o-DNB. Plasmid pAW42, containing only the 3.3-kb BamHI fragment, permitted cells to tolerate elevated o-DNB concentrations. Further subcloning established that the 2-kb HindII fragment contained within pAW47 was sufficient to provide elevated o-DNB resistance. To gain insight into the nature of the ROD1 gene product, we determined the DNA sequence around this HindII fragment.

DNA sequence analysis revealed a single large open reading frame of 837 amino acids with a calculated molecular mass of 92 kDa. Several TATA-like sequences are present upstream of ROD1 and a potential transcription termination sequence (TATA) (29) is located from 2592 to 2597 in the 3' non-coding region, 77 base pairs downstream of the translation termination codon. No good candidates for yAP-1 recognition elements were found in the ROD1 5' flanking region.

Rod1p is rich in serine and asparagine residues containing 15 and 9\%, respectively, of these amino acids. Computer search indicated that other than similarities based on the high content of these two amino acids, Rod1p did not show significant similarity to any proteins of known function in the data base. ROD1 has also been sequenced as a gene of unknown function present on chromosome XV as part of the effort to sequence the yeast genome (GenBank\textsuperscript{TM} accession number: X87331). However, striking sequence similarity was found between Rod1p and a protein of unknown function identified on chromosome IV during the sequencing of this chromosome. This locus was designated YFR022W and shares 43\% sequence identity with Rod1p throughout the length of both protein chains (Fig. 3). The most extensive sequence identity is in the amino-terminal segments of these two different proteins. A more distantly related protein encoded on chromosome II (YBL101c) was also found to exhibit significant sequence similarity to Rod1p (20\%), but no functional information is available for this factor.

Computer analysis of the Rod1p sequence indicated that this protein was highly basic with a net charge of +10 at pH 7. Hydropathy analysis of Rod1p indicated that the 350 amino acids at the COOH terminus are predominantly hydrophilic, while the NH\(_{2}\)-terminal three-fifths of the protein exhibited a more hydrophobic character. Analysis of potential transmembrane segments using the Protean program of the Lasergene sequence analysis package (DNAstar, Inc.) indicated that one segment of Rod1p (246–263) might be able to serve as a membrane spanning domain. Twelve potential N-linked glycosylation sites (Asn-X-Ser/Thr) (30) are found in the COOH-terminal region, although these may occur due to the high asparagine and serine content of the factor. At least in the context of the Rod1p-Myc fusion protein (see below), we were not able to detect any endoglycosidase H-sensitive molecular mass forms of the protein (data not shown).

Immunodetection of the ROD1 Gene Product—In order to determine if yeast cells produce a polypeptide corresponding to the protein predicted by the ROD1 DNA sequence, a Rod1p-Myc fusion protein was constructed. 115 amino acids from the COOH terminus of Rod1p were replaced by synthetic oligonucleotides encoding an antigenic determinant from the c-Myc proto-oncoprotein. The 2\( \mu \)m plasmid pAW75 carrying this fusion construct was transformed into wild-type cells and tested for o-DNB resistance. The epitope-tagged version of the ROD1 gene still conferred resistance to o-DNB (Fig. 4). This result indicated that the epitope-tagged Rod1p still retained the function of the overproduced but untagged protein.

Whole cell protein extracts were prepared from wild-type cells carrying either pAW49 or pAW75. Western blot analysis of these extracts was carried out using the anti-Myc monoclonal antibody 9E10. A polypeptide with a molecular mass of approximately 100 kDa was detected in crude extracts from cells expressing the 2\( \mu \)m Rod1p-Myc fusion construct but not from cells producing the untagged Rod1p (Fig. 4A). The predicted mass of the Myc-tagged Rod1p-Myc was 81 kDa. The
cause of this discrepancy in the expected and observed molecular masses is not known but may be due to the unusual amino acid composition of this protein.

Subcellular Localization of Rod1p-Myc—To determine where Rod1p was likely to act in the cell, subcellular fractionation by differential centrifugation of total protein extracts was carried out. Rod1p was found in the particulate fraction (P100) (Fig. 5). No immunoreactivity was detected in the high speed 100,000 g supernatant fraction (S100). The possible membrane association of Rod1p-Myc was further investigated using a variety of extraction conditions to attempt to solubilize the protein from the P100 fraction. Protein extracts were subjected to different treatments known to solubilize membrane-associated proteins. After these treatments, each extract was separated into P100 and S100 fractions by centrifugation. The presence of Rod1p-Myc was detected by immunoblotting with the anti-Myc antibody. This analysis indicated that neither treatment with alkaline carbonate buffer (0.1 M Na2CO3 (pH 11)) nor the non-ionic detergent Triton X-100 was able to release significant amounts of Rod1p. As a control, the known plasma membrane-associated yeast proteins Ras1p and Ras2p were examined. Treatment with alkaline carbonate buffer did not release detectable amounts of Ras, whereas Triton X-100 efficiently solubilized Ras from total membranes. To compare the behavior of Rod1p with a known cytoskeleton protein, fractionation of yeast S. cerevisiae protein was examined on the same blot using an anti-tubulin antibody. The analysis indicated that the tubulin protein stays in the supernatant fraction (S10 and S100) after 10,000 g and 100,000 g centrifugation.

To investigate the possibility that Rod1p might be a plasma membrane protein, differential subcellular fractions from cells carrying the pAW75 plasmid (Rod1p-Myc), enriched either in soluble portions, in mitochondria and ribosomal components, or in plasma membranes, were analyzed. Fig. 6 shows that in both the crude membrane fraction and plasma membrane-enriched fraction (P100), Rod1p-Myc fusion protein was detected. Ras, a known plasma membrane-associated protein, was found in the same fractions after immunoblotting, while the intracellular protein carboxypeptidase Y was de-
ROD1, a New S. cerevisiae Multiple Resistance Locus

Fig. 5. Subcellular fractionation of Rod1p-Myc. A crude protein extract was prepared from SEY6210 cells carrying a plasmid expressing the Rod1p-Myc fusion protein. This crude extract was resolved into particulate (P) or soluble (S) fractions by differential centrifugation at 10,000 × g (P100 and S100) or 100,000 × g (P100 and S100). Prior to centrifugation, the extracts were treated with lysis buffer, 0.1 M Na2CO3, or Triton X-100 (TX-100). Aliquots of each sample were electrophoresed on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blot analysis. The filter was probed with antibodies directed against the Myc epitope (Rod1p-Myc), Ras (Ras), or tubulin protein (TUB). The location of molecular mass markers is shown on the left-hand side of the figure.

Fig. 6. Plasma membrane fractions are enriched for Rod1p-Myc. SEY6210 cells were broken by glass bead lysis and separated into particulate (P100) and soluble (S100) fractions by centrifugation at 100,000 × g. The P100 fraction was then acid-extracted as described (3) to generate a mitochondrial/ribosome-enriched (MIT-RIB) fraction and plasma membrane-enriched (PL-MEM) fraction. Aliquots of each sample were electrophoresed on SDS-polyacrylamide gel electrophoresis and subjected to Western analysis. The antibodies used to visualize the locations of the indicated proteins are described in the legend to Fig. 5 with the exception of a mouse monoclonal directed against carboxypeptidase Y (CPY). Molecular mass markers are indicated on the left-hand side of the figure.

Fig. 7. Overlapping pleiotropic phenotypes of rod1 and yap1 mutants. Yeast strains were constructed that lacked the ROD1 (YAW14) gene or both the Rod1p and YAP1 loci (YAW15). These strains were assayed by spot test analysis (20) along with isogenic wild-type (SEY6210) and Δyap1 (SM10) cells. Strains were tested on YPD medium containing the indicated concentrations of inhibitors and were photographed according to the pattern shown on the top of the figure. Bars indicate the two different inhibitor concentrations tested for o-DNB, diamide, and CaCl2.

from −76 to +1330 base pairs, including the ROD1 translational start codon, was replaced by a hisG-URA3-hisG fragment. The resulting plasmid, pAW60, bearing a disrupted ROD1 gene, was transformed into the wild-type strain (SEY6210) following a BamHI restriction digest. Stable URA− transformants were obtained. Next, the URA− transformants were treated with 5-fluoro-orotic acid to remove the URA3 gene as described (23). Genomic Southern blot indicated that the viable URA− colonies contained a single disrupted chromosomal copy of the ROD1 gene (data not shown). The resulting Δrod1 strain was designated YAW14.

There was no detectable difference in growth between wild-type and Δrod1 cells in the absence of a toxic agent in the medium. This finding suggests that Rod1p is not an essential gene. However, several differences in growth between the wild-type and Δrod1 mutant strains were observed on YPD plates containing o-DNB, zinc, diamide, or calcium (Fig. 7). The Δrod1 mutant strain was unable to grow on plates containing 200 μM o-DNB or 10 mM ZnCl2, while the wild-type strain was still able to grow at these concentrations. In addition, the Δrod1 strain grows slower than wild-type in medium containing 12 mM diamide or 10 mM CaCl2. These findings indicated that Rod1p plays a physiological role in resistance to o-DNB, zinc, diamide, and calcium. These data also indicate that the role of Rod1p in drug resistance is not limited to o-DNB tolerance.

Since both Δyap1 and Δrod1 strains were hypersensitive to o-DNB, zinc, and diamide, we wanted to further examine the overlap in resistance between these two genes. To accomplish this, a strain lacking both these loci (Δrod1Δyap1) was constructed. This strain was designated YAW15. The Δyap1Δrod1 strain (YAW15) was dramatically more sensitive to o-DNB and calcium than either Δyap1 or Δrod1 cells. This finding indicates that the products of these loci are likely to physiologically contribute to o-DNB and calcium tolerance. The Δyap1Δrod1
strain was slightly more sensitive to diamide than cells lacking the YAP1 gene, although YAP1 was clearly the major resistance determinant. In contrast, a strain lacking the ROD1 gene was quite sensitive to zinc, but this sensitivity was not detectably enhanced in the Δyap1/Δrod1 background. These data indicate that the functions of YAP1 and ROD1 are additive and contribute roughly equally in terms of resistance to some agents (o-DNB, calcium). However, YAP1 contributes more to diamide tolerance, and ROD1 has the major effect on zinc resistance.

Epistatic Analysis of ROD1 and YAP1—We tested the ability of high copy plasmids expressing Rod1p or YAP1 to suppress the phenotypes caused by lack of the structural gene encoding the other factor. The ability of the high copy ROD1 gene to suppress the phenotypes produced in a Δyap1 strain was examined (Fig. 8A). A 2-μm plasmid carrying the ROD1 gene was transformed along with the 2-μm vector as a control, into an isogenic pair of strains differing only in the presence of a normal YAP1 locus. Additionally, a high copy YAP1-containing plasmid was transformed into an analogous pair of ROD1 and rod1-11::hisG strains (Fig. 8B). Selected transformants were then tested for growth in the presence of o-DNB, zinc, and calcium.

High copy ROD1 was only able to elevate o-DNB and zinc tolerance normally in the presence of an intact copy of chromosomal YAP1. In opposition to this YAP1 dependence of the ROD1 effects on o-DNB and zinc, loss of YAP1 had no effect on high copy ROD1-mediated calcium resistance. The 2-μm YAP1 plasmid did not elevate o-DNB or calcium resistance in a strain lacking the ROD1 gene. However, high copy YAP1-mediated zinc tolerance was not dependent on the presence of ROD1. These data are consistent with the belief that Rod1p and YAP1 each contribute separate functions to o-DNB resistance with both of these functions necessary for normal tolerance to result. However, the relationship of Rod1p and YAP1 to zinc and calcium resistance phenotypes suggests a more complicated interaction of these two gene products. This analysis clearly shows that Rod1p and YAP1 are both key participants in the cellular handling of several different toxic compounds. Additionally, assay of GST present in ROD1 high copy transformants indicated that the activity of this enzyme was not elevated relative to a vector control (data not shown). This observation strongly suggests that the effect of Rod1p on drug resistance does not come about through an increase in GST activity.

**DISCUSSION**

Previous studies on pleiotropic drug resistance of yeast have provided information about several different gene products involved in allowing cells to deal with cytotoxic agents in their environment (reviewed in Ref. 2). Most of these loci have been integral membrane transport proteins and transcription factors. In the work described here, we provide an example of a new type of resistance determinant that does not obviously fit into these two well-known classes of gene products.

Numerous studies in several laboratories have established that elevating the copy number of YAP1 increases resistance to cycloheximide, 1,10-phenanthroline, cadmium, H2O2, and numerous other agents (20, 31–33). Our observation that high copy ROD1 does not elevate tolerance to all the same drugs as YAP1 suggests that Rod1p is not likely to act through YAP1. Furthermore, Northern blot and lacZ gene fusion experiments have established that ROD1 is not under the transcriptional control of YAP1.2 These data are consistent with the belief that Rod1p acts to contribute separate functions to several YAP1-mediated resistances.

The details of how Rod1p acts are still unknown, but this factor clearly provides a limiting function for o-DNB, calcium, and zinc resistance. Loss of ROD1 causes cells to become hypersensitive to these compounds, while overproduction of Rod1p elevates resistance to each of these agents. The hypersensitivity elicited by loss of ROD1 was exacerbated with concomitant removal of YAP1, with the exception of zinc resistance. Disrupting YAP1 in a Δrod1 background does not further reduce zinc tolerance while overproduction of YAP1 completely suppresses the need for ROD1. These data suggest that Rod1p is necessary for physiological zinc homeostasis, while high levels of YAP1 can bypass this requirement. This situation is reminiscent of the effect of YAP1 on cycloheximide resistance. Overproduction of YAP1 leads to high level cycloheximide tolerance, but a Δyap1 strain is no more sensitive to cycloheximide than a wild-type strain (34).

Both YAP1 and Rod1p are required for o-DNB resistance irrespective of the level of either factor. However, while both YAP1 and ROD1 are necessary for calcium resistance when these genes are present in single copy, the presence of ROD1 on a high copy plasmid eliminates the need for YAP1. This observation suggests that the mechanism of resistance to o-DNB and calcium is not likely to be the same, since there are different genetic requirements for YAP1 and Rod1p to effect tolerance to these compounds.

The localization information provided here provides a framework for beginning to understand the action of Rod1p. Our data suggests that Rod1p is associated with the plasma membrane fraction of yeast cells, but further experimentation is necessary to confirm this suggestion. Attempts to localize the Rod1p-Myc fusion protein by immunofluorescence were not successful, possibly owing to the finding that this fusion protein was extremely sensitive to zymolyase treatment (data not shown). A similar sensitivity has been noted for the plasma membrane protein Ste6p (35). This proteolytic sensitivity of Rod1p-Myc is consistent with the enhanced accessibility of the fusion protein that could occur if the factor was exposed to the outside of the cell. The possibility that Rod1p is exposed to the outside of the

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2 A. Wu, unpublished data.
S. cerevisiae cell is being investigated. This work establishes several new facts about drug resistance in S. cerevisiae. First, production in S. cerevisiae of a fungal GST protein from I. orientalis is capable of elevating resistance to at least one toxic agent (o-DNB). Second, S. cerevisiae possesses a locus designated ROD1 that is required, along with the transcription factor yAP-1, for normal o-DNB tolerance. Additionally, Rod1p enhances the ability of the cell to tolerate several compounds known to be under the phenotypic influence of yAP-1. However, Rod1p only affects a small subset of the large number of resistance phenotypes influenced by yAP-1, indicating a much narrower range of actions for Rod1p. Finally, the ROD1 gene product fractionates in the particulate component of S. cerevisiae, unlike the soluble yAP-1 protein (data not shown). Understanding the action of Rod1p will provide important new information about how this novel protein influences drug resistance in eukaryotes.

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REFERENCES
1. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427
2. Balzi, E., and Goffeau, A. (1995) J. Bioenerget. Biomem. 27, 71–76
3. Balzi, E., Wang, M., Leterme, S., Van Dyck, L., and Goffeau, A. (1994) J. Biol. Chem. 269, 2206–2214
4. Bissinger, P. H., and Kuchler, K. (1994) J. Biol. Chem. 269, 2206–2214
5. Hirata, D., Yano, K., Miyahara, K., and Miyakawa, T. (1994) Curr. Genet. 26, 285–294
6. Moye-Rowley, W. S., Harshman, K. D., and Parker, C. S. (1989) Genes & Dev. 3, 283–292
7. Leonard, P. J., Rathod, P. K., and Golin, J. (1994) Antimicrob. Agents Chemother. 38, 2492–2494
8. Wu, A., and Moye-Rowley, W. S. (1994) Mol. Cell. Biol. 14, 5832–5839
9. Wemmie, J. A., Szczypka, M. S., Thielle, D. J., and Moye-Rowley, W. S. (1994) J. Biol. Chem. 269, 32592–32597
10. Kuge, S., and Jones, N. (1994) EMBO J. 13, 655–664
11. Ohtake, Y., Satou, A., and Yabuuchi, S. (1990) Agric. Biol. Chem. 54, 3145–3150
12. Tew, K. D. (1994) Cancer Res. 54, 4313–4320
13. Pickett, C. B., and Lu, A. Y. H. (1989) Annu. Rev. Biochem. 58, 743–764
14. Tamaki, H., Kumagai, H., and Tochikura, T. (1991) Biochem. Biophys. Acta 1089, 276–279
15. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
16. Sherman, F., Fink, G., and Hicks, J. (1979) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Kumagai, H., Tamaki, H., Koshino, Y., Suzuki, H., and Tochikura, T. (1988) Agric. Biol. Chem. 52, 1377–1382
18. Carlson, M., and Botstein, D. (1982) Cell 28, 145–154
19. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425
20. Wu, A., Wemmie, J. A., Edgington, N. P., Guevara, J. L., and Moye-Rowley, W. S. (1993) J. Biol. Chem. 268, 18850–18858
21. Rothstein, R. (1991) Methods Enzymol. 194, 281–301
22. Alani, E., Cao, L., and Kiedron, N. (1987) Genetics 116, 541–545
23. Boeke, J. D., Lacroute, F., and Fink, G. R. (1984) Mol. & Gen. Genet. 197, 345–356
24. Kolodziej, P. A., and Young, R. A. (1991) Methods Enzymol. 194, 508–519
25. Franzusoff, A., Rothblatt, J., and Schekman, R. (1991) Methods Enzymol. 194, 662–674
26. Graham, T. R., Seeger, M., Payne, G. S., Mackay, V. L., and Emr, S. D. (1994) J. Cell Biol. 127, 667–678
27. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
28. Tamaki, H., Kumagai, H., and Tochikura, T. (1990) Biochem. Biophys. Res. Commun. 172, 669–675
29. Guo, Z., Russo, P., Yun, D.-Y., Butler, J., and Sherman, F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4211–4214
30. Orlean, P., Kuranda, M. J., and Albright, C. F. (1991) Methods Enzymol. 194, 682–697
31. Bossier, P., Fernandes, L., Rocha, D., and Rodrigues-Pousada, C. (1993) J. Biol. Chem. 268, 23640–23645
32. Schnell, N., Kremz, B., and Entian, K.-D. (1992) Curr. Genet. 21, 269–273
33. Hertle, K., Haase, E., and Brendel, M. (1991) Curr. Genet. 19, 429–433
34. Dexter, D., Moye-Rowley, W. S., Wu, A.-L., and Golin, J. (1994) Genetics 136, 505–515
35. Kuchler, K., Dohlmans, H. G., and Thoner, J. (1993) C. Cell Biol. 120, 1203–1215
36. Deveraux, J., Haeberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395