Identification of \textit{Saccharomyces cerevisiae} Isoleucyl-tRNA Synthetase as a Target of the \textit{G}_{1}-specific Inhibitor Reveromycin A*  

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Yuji Miyamoto‡, Kiyotaka Machida§, Masaki Mizunuma‡, Yuji Emoto‡, Naomi Sato‡, Kohji Miyahara‡, Dai Hirata‡, Takeo Usui§, Hitotoshi Takahashi§, Hiroyuki Osada§, and Tokichi Miyakawa‡†  

From the ‡Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima 739-8530, Japan and the §Antibiotics Laboratory, RIKEN, Hirono 2-1, Saitama 351-0198, Japan  

To dissect the action mechanism of reveromycin A (RM-A), a \textit{G}_{1}-specific inhibitor, a \textit{Saccharomyces cerevisiae} dominant mutant specifically resistant to RM-A, was isolated from a strain in which the genes implicated in nonspecific multidrug resistance had been deleted. The mutant gene (\textit{YRR2–1}) responsible for the resistance was identified as an allele of the \textit{ILS1} gene encoding tRNA\textsuperscript{Ile}\textsuperscript{5s} synthetase (IleRS). The activity of IleRS, but not several other aminoacyl-tRNA synthetases examined in wild type cell extract, was highly sensitive to RM-A (IC\textsubscript{50} = 8 ng/ml). The IleRS activity of the \textit{YRR2–1} mutant was 4-fold more resistant to the inhibitor compared with that of wild type. The mutation IleRS\textsubscript{N660D}, near the KMSKS consensus sequence commonly found in the class I aminoacyl transferases, was found to be responsible for RM-A resistance. Moreover, overexpression of the \textit{ILS1} gene from a high-copy plasmid conferred RM-A resistance. These results indicated that IleRS is a target of RM-A in vivo. A defect of the \textit{GCN2} gene led to decreased RM-A resistance. IleRS inhibition by RM-A led to transcriptional activation of the \textit{ILS1} gene \textit{via} the Gen2-Gen4 general amino acid control pathway, and this autoregulation seemed to contribute to RM-A resistance.  

Reveromycin A (RM-A)\textsuperscript{1} (see Fig. 1), which was discovered in the culture medium of \textit{Actinomycetes} due to its inhibitory activity on the epidermal growth factor-dependent responses of mouse epidermal cells, blocks the cell division cycle of mammalian cells in \textit{G}_{1} phase (1,2). However, the mechanism by which RM-A elicits the cell-cycle-specific growth arrest in mammalian cells is unknown. Yeast provides an ideal system to investigate the diverse biological activity of drugs because of the observations that various drugs that are effective to both yeast and higher eukaryotic cells often elicit their effects by an identical mechanism, sharing structurally and functionally conserved drug target molecules. Because RM-A exhibits an antifungal activity, we attempted to dissect its mode of action by identifying its target molecule by the power of yeast genetics (1, 2).  

Our previous genetic approaches in \textit{Saccharomyces cerevisiae} have demonstrated that the ATP-binding cassette superfamily anionic drug transporter Yrs1/Yor1 and the Zn(II)$_{2}$Cy$_{5g}$-type transcription factor Yrr1 are important for RM-A resistance by enhancing the Yor1-mediated multidrug resistance mechanism (2–4). However, the molecular mechanism by which RM-A inhibits the growth of yeast cells has still remained unknown. On the basis of these findings, the purpose of this study is to identify as yet unknown RM-A target molecule in yeast by isolating and characterizing mutants resistant to RM-A in a genetic background in which several genes implicated in multidrug resistance have been deleted. We revealed that a dominant mutation (\textit{YRR2–1}) in the \textit{ILS1} gene encoding isoleucyl-tRNA synthetase (IleRS) is responsible for the RM-A resistance. The IleRS activity, but not those of several other aminoacyl-tRNA synthetases examined in the wild type (WT) cell extract, was highly sensitive to RM-A, whereas the IleRS activity in the \textit{YRR2–1} strain was more resistant to the inhibitor, indicating that IleRS is a major target for RM-A in yeast. The transcription of \textit{ILS1} gene is activated by the Gen2-Gen4 general amino acid control pathway (for review, see Ref. 5). We also demonstrated that a defect of the \textit{GCN2} gene led to an increased RM-A sensitivity, indicating that the elevation of \textit{ILS1} expression by the Gen2-Gen4-mediated autoregulatory mechanism also contributes to RM-A resistance.  

Materials and Methods  

\textit{Strains, Plasmids, and Media—} \textit{S. cerevisiae} strain W303–1A (MAT\textit{a} trp1 leu2 his3 ura3 ade2 can1–100) and its derivatives were used throughout this study. Strain MLC30, a \textit{yrr1::HIS3} \textit{S}_{\text{yrr1}}::\textit{TRP1} \textit{Δpdr1::hisG-URA3-hisG} \textit{Δpdr3::hisG-URA3-HisG} quadruplex disruption derivative, was constructed by crossing MLC15–2 (MAT\textit{a} \textit{S}_{\text{yrr1}}::\textit{TRP1} leu2 his3 \textit{Δpdr1::hisG-URA3-hisG} \textit{Δpdr3::hisG-URA3-HisG} ade2 can1–100) and MLC26–1C (MAT\textit{a} trp1 leu2 \textit{S}_{\text{yrr1}}::HIS3 ura3 ade2 can1–100), followed by tetrad analysis of spores derived from the diploid (2, 3). Yeast strains were grown on YPD medium at 28 °C. Solid YPD medium containing RM-A was prepared by adding an appropriate amount of RM-A from a stock solution (1 mg/ml in water) to autoclaved YPD agar medium that was adjusted at pH 4.5 using 1 N HCl before autoclaving (2).  

\textit{Drug Sensitivity Test—}The effect of RM-A or other drugs on the growth of yeast strains was compared by spotting cell suspension on solid medium. A 3-fold serial dilution of yeast cells suspended in water (\textasciitilde 2 \times 10^{6} \text{cells/ml}) was applied on YPD solid medium containing various concentrations of drugs using an applicator. The plates were incubated at 28 °C for 2–3 days.  

\textit{[35S]Methionine Incorporation—} Strains were grown to log phase in
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RESULTS

Physiological Effect of RM-A on Yeast—The RM-A treatment of mammalian cells blocks both protein synthesis and cell growth, accumulating G1 cells (1). We first investigated the effect of RM-A on protein synthesis in yeast by assaying the incorporation of [35S]methionine into proteins in the presence of the drug. The cells were treated with RM-A (0.6 μg/ml) for various periods of time at a concentration that inhibits cell growth. The immediate cessation of protein synthesis by the drug treatment suggested that RM-A is a potent inhibitor of protein synthesis (data not shown). We next examined the effect of RM-A on cell cycle progression by fluorescence-activated cell sorting analysis and budding indexes. The results of fluorescence-activated cell sorting analysis showed that the cells with 1C DNA content accumulated when the cells were cultivated with RM-A (Fig. 2A). Consistent with this data, the population of the unbudded cells after 6 h of RM-A treatment increased from 20 to 57% (Fig. 2B). These results indicated that RM-A treatment of yeast cells blocks the cell cycle in G1.

Isolation and Genetic Analysis of Dominant RM-A-Resistant Mutants—If the interaction between the drug and its cellular target is impaired by mutation, it would lead to a dominant phenotype specifically resistant to the drug. Based on this assumption, to dissect the mode of RM-A action in yeast, we performed a screen for the mutants that exhibited dominant RM-A resistance. Our previous studies in S. cerevisiae have demonstrated that the YRS1/YOR1 gene encoding an ATP-binding cassette superfamily drug transporter protein and the YRR1 gene encoding a Zn(II)2Cys6-type transcription factor are important for RM-A resistance, and that a dominant YRR1 mutation caused the elevation of the YOR1 transcription leading to RM-A resistance (2–4). Two homologous genes, PDR1 and PDR3, encode major transcription factors that regulate the expression of various genes that function in the multidrug resistance (for review, see Ref. 9). Some mutations in YRR1, PDR1, and PDR3 genes confer dominant drug resistance through the overexpression of the drug transporters (3, 9–11). To eliminate the resistance that arises by mutations in the previously characterized genes implicated in multidrug resistance, we constructed a parental strain in which YRR1, PDR1, and PDR3 genes, together with the YOR1 gene, had been deleted. The growth of the Δyrr1 Δpdr1 Δpdr3 Δyor1 quadruple deletion mutant (MLC30) was hypersensitive to RM-A (Fig. 3A). To isolate spontaneous RM-A-resistant mutants, exponentially growing cells were spread on YPD agar plates.
containing 0.7 μg/ml RM-A at a cell density of about $1 \times 10^7$ cells per plate. Colonies formed after 3 days of incubation at 28 °C were picked up, each strain was crossed with WT haploid strain, and the resulting diploids were tested for RM-A resistance. Of the 12 mutants isolated, six exhibited a dominant phenotype for the resistance. By tetrad analysis of the spores derived from these six diploid strains, five strains exhibited a 2:2 segregation pattern for RM-A resistance, indicating a single mutation. Complementation analysis was performed by crossing among the haploid strains. The results of tetrad analysis indicated that all five mutants belong to a single complementation group designated YRR2 (for yeast reveromycin A resistance). The resistance conferred by the YRR2 mutations was highly specific to RM-A and exhibited no significant cross-resistance to various other drugs examined, which included cycloheximide and tautomycin (data not shown).

Cloning and Identification of the YRR2–1 Mutant Gene—To investigate the role of the YRR2 gene product in RM-A drug action, we cloned and characterized the YRR2 gene. For gene cloning, we first tested if the YRR2–1 dominant mutants may exhibit a recessive phenotype useful for cloning from a WT genomic library (e.g. high- or low-temperature sensitivity). However, because no appropriate phenotype was found, we decided to isolate the YRR2 mutant gene (YRR2–1) from a mutant genomic library constructed with YCp50 vector, and this library was used to transform the MLC30 strain. The transformants were selected for the ability to grow on a plate containing 0.7 μg/ml RM-A. Two plasmids designated pYM2–1 and pYM2–2 were recovered. Partial sequencing of an overlapping fragment of the inserts revealed that they contained AUT7 and ILS1 genes and the YBLO77W open reading frame (data not shown). Subcloning of the fragments indicated that the ILS1 gene, which encodes IleRS belonging to class I aminoacyl-tRNA synthetases, is responsible for the resistance (12). Linkage of the YRR2–1 mutation to the ILS1 locus was verified by tetrad analysis of the spores derived from the diploid produced by crossing WT strain and an integrative transformant with a LEU2 marker at the ILS1 locus (data not shown). The wild type YRR2–1 allele was screened from a YCp50-based genomic DNA library of the W303–1A strain by the colony hybridization method. By comparing the amino acid sequences of the mutant IleRS with that of WT, two mutations, G646A and N660D, were revealed near the KMSKS consensus sequence commonly found in the class I aminoacyl transferases (13). Of these mutations, N660D was found to be responsible for the RM-A resistance by site-directed mutagenesis (Fig. 4A). The Aaa-660 residue is conserved between yeast and human IleRS (14).

Disruption of the ILS1 gene led to a lethal phenotype as previously revealed by the whole genome approach of yeast (15) (data not shown). Consistent with the notion that IleRS is a target of RM-A action, overexpression of ILS1 (WT) from a 2μ-based high-copy number plasmid conferred strong RM-A resistance (Fig. 4B).

IleRS Is Sensitive to RM-A in Vitro—Because IleRS was suggested to be an in vivo target for RM-A, we next examined if IleRS is sensitive to RM-A in vitro. Cell-free extract prepared from WT strain was assayed for the activity to catalyze the transfer of 3H-labeled isoleucine onto tRNA in the presence of various concentrations of RM-A (0, 10, 100, and 1,000 ng/ml). For comparison, the activities of several other aminoacyl-tRNA synthetases were assayed using radiolabeled leucine, valine, methionine, glutamic acid, and phenylalanine as substrate. Aminoacyl synthetases for methionine, leucine, and valine belong to type I aminoacyl synthetases. As shown in Fig. 5A, the IleRS activity decreased to about 20% of control in the presence of 10 ng/ml RM-A, a concentration almost two orders of magnitude below that for the growth inhibition. In contrast, the aminoacyl transferases for leucine, valine, methionine, glutamic acid, and phenylalanine were virtually insensitive to RM-A. These results indicated that IleRS, among various other aminoacyl-tRNA synthetases, is extremely sensitive to RM-A. The considerable difference in the inhibitory concentrations in vivo (growth inhibition) and in vitro (IleRS inhibition) is likely to reflect the poor permeability of the ionizing drug RM-A across the membrane (1).

The YRR2–1 Mutation Causes IleRS with Increased RM-A Resistance—Next, we compared RM-A sensitivity of IleRS from WT and YRR2–1 strains in vitro (Fig. 5B). In the absence of the inhibitor, the specific activities of IleRS from the two strains were comparable (data not shown). However, IleRS from the
mutant was more resistant to RM-A than that from the WT strain. The IC$_{50}$ values for IleRS from the WT and YRR2–1 strains were 8 and 32 ng/ml, respectively, showing that the mutant enzyme was 4-fold more resistant to the inhibitor. It was confirmed by the in vitro assay that IleRS$^{N660D}$, but not IleRSG646A, mutation was responsible for the RM-A resistance (Fig. 5B).

Defect of GCN2 Leads to RM-A Sensitivity—Previously, we isolated and characterized the yeast mutants that exhibited increased RM-A sensitivity (2). This study identified the genetic complementation groups yrs2, -3, and -4 in addition to the previously characterized gene yrs1 that encodes a multidrug resistance-associated protein-type ATP-binding cassette transporter responsible for RM-A resistance (2,5). Of these RM-A-sensitive mutants, the yrs2 mutation was identified as a mutant allele of the GCN2 gene encoding a protein kinase that positively regulates GCN4 expression. The GCN4 gene encodes a transcriptional activator for various amino acid biosynthetic genes implicated in general amino acid control in response to starvation for single amino acids (for review, see Ref. 5). In fact, the Δgcn2 strain exhibited increased RM-A sensitivity (Fig. 6A). Moreover, in accordance with the idea that ILS1 is a downstream component of GCN2, a gene contained in a low-copy suppressor of the RM-A sensitivity of the yrs2/gcn2 mutation was identified as the ILS1 gene (data not shown). Furthermore, Northern blot analysis of ILS1 mRNA revealed that the ILS1 expression level was elevated during RM-A treatment in a partially GCN2-dependent manner (Fig. 6B). However, the

DISCUSSION

The genetic and biochemical approaches on the yeast RM-A resistance have revealed that IleRS is a target of RM-A drug action. The physiological effects of RM-A on yeast, such as the inhibition of growth and protein synthesis, can be explained by the essential role of IleRS for growth and protein synthesis. Of several aminoacyl-tRNA synthetases examined in vitro, only IleRS was highly sensitive to RM-A. Furthermore, both the dominant YRR2–1 mutation and overexpression of ILS1 (WT) from a high-copy plasmid conferred resistance specifically to RM-A, and only the ILS1 mutant alleles were found among the dominant RM-A-resistant mutations. Based on these results, we concluded that IleRS is a major in vivo target of RM-A action in yeast. The molecular basis for the extreme specificity of RM-A to IleRS still remains to be clarified.
Under starvation conditions, when nutrients are limited, *S. cerevisiae* cells accumulate in G1 (16, 17) (for review, see Ref. 18). When nutrients are limiting, haploid yeast cells do not proceed to START in late G1, but instead exit the mitotic cell cycle in early G0 and enter a stationary or G0 phase. The cell-cycle-specific growth inhibition may be the result of the cessation of protein synthesis and thereby leads to physiological changes characteristic of starved cells entering stationary phase (G0). In fact, we found by reciprocal shift experiments that the RM-A restriction point was in early G1 before the α-factor arrest point (data not shown). A similar mode of growth inhibition was reported with rapamycin, which ultimately blocks translational initiation (19). As suggested for rapamycin action, the cell-cycle-specific growth inhibition by RM-A may be elicited by the decreased translation of an unstable protein(s) essential for G1 progression, such as G1 cyclins, which are unstable and limiting in G1 (19). The entry into the stationary phase enables cells to maintain viability for long periods when nutrients are not available.

Our genetic approach to the mode of RM-A action in yeast revealed that the GCN2 gene plays a role in RM-A resistance. The expression of the ILS1 gene is under the control of the GCN2–GCN4 pathway implicated in general amino acid control that responds to starvation for single amino acids in a manner dependent on Gen2 (for review, see Ref. 5). A simple interpretation for the involvement of Gen2 in RM-A resistance is that RM-A treatment leads to a decreased level of charged tRNAile, which in turn activates Gcn2 kinase (probably via the Gcn2p HisRS-like domain sensing uncharged tRNAs), and thereby induces the ILS1 expression via the GCN2–GCN4 pathway. In fact, the transcription of the ILS1 gene was activated in response to RM-A stress (Fig. 6, A and B). Gcn2 phosphorylates the α subunit of the translation initiation factor-2 (eIF2-α) leading to the inhibition of protein synthesis and thereby mediates translational control of GCN4 (20). The feedback regulation mediated by the GCN2–GCN4 pathway is reminiscent of the autoregulatory model described for the yeast GCD5/KRS1 gene, which encodes lysyl-tRNA synthetase. In this model, the gcd5–1 mutation causes a reduced charging of tRNAlys,

In this study, the dominant mutants specifically resistant to RM-A were the key to the identification of the drug target molecule. Dominant multidrug resistance is known to arise in the genes that encode Pdr1, Pdr3, and Yrr1 transcription factors in the presence of drugs (3, 9–11). In this regard, the use of the genetic background in which various genes implicated in nonspecific multidrug resistance had been eliminated was effective, as indicated by the result that all five mutations that exhibited a dominant resistance phenotype fell into a single genetic complementation group YRR2/ILS1. Analogous approaches would be useful in dissecting the mode of drug action with other drugs of biological or pharmacological interest. For gene cloning, we constructed and screened a genomic library of the YRR2–I mutant because the appropriate recessive phenotype for screening from the WT genomic library was not available with the mutation. However, if a recessive phenotype, such as low- or high-temperature sensitivity, that facilitates gene cloning using a WT genomic library is linked to the dominant mutation, this approach would become more useful for the identification of a drug target molecule.

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