Mutations in Ribosomal Protein L10e Confer Resistance to the Fungal-specific Eukaryotic Elongation Factor 2 Inhibitor Sordarin*

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Michael C. Justice‡, Theresa Ku‡, Ming-Jo Hsu‡, Karen Carniol§, Dennis Schmatz‡, and Jennifer Nielsen‡¶

From the §Department of Basic Animal Science Research, Merck Research Laboratories, Rahway, New Jersey 07065 and ¶Wesleyan University, Middletown, Connecticut 06459

The natural product sordarin, a tetracyclic diterpene glycoside, selectively inhibits fungal protein synthesis by impairing the function of eukaryotic elongation factor 2 (eEF2). Sordarin and its derivatives bind to the eEF2-ribosome-nucleotide complex in sensitive fungi, stabilizing the post-translocational GDP form. We have previously described a class of Saccharomyces cerevisiae mutants that exhibit resistance to varying levels of sordarin and have identified amino acid substitutions in yeast eEF2 that confer sordarin resistance. We now report on a second class of sordarin-resistant mutants. Biochemical and molecular genetic analysis of these mutants demonstrates that sordarin resistance is dependent on the essential large ribosomal subunit protein L10e in S. cerevisiae. Five unique L10e alleles were characterized and sequenced, and several nucleotide changes that differ from the wild-type sequence were identified. Changes that result in the resistance phenotype map to 4 amino acid substitutions and 1 amino acid deletion clustered in a conserved 10-amino acid region of L10e. Like the previously identified eEF2 mutations, the mutant ribosomes show reduced sordarin-conferred stabilization of the eEF2-nucleotide-ribosome complex. To our knowledge, this report provides the first description of ribosomal protein mutations affecting translocation. These results and our previous observations with eEF2 suggest a functional linkage between L10e and eEF2.

Eukaryotic elongation factor 2 (eEF2)1 and its prokaryotic counterpart, elongation factor G (EF-G), promote the translocation of the ribosome along messenger RNA during the elongation phase of protein synthesis. Hydrolysis of GTP to GDP drives translocation and is associated with a presumed conformational change in eEF2. Sordarin (1) and its analogs are fungal-specific translation inhibitors (2, 3) that bind to the eEF2-ribosome-GDP complex in Saccharomyces cerevisiae, stabilizing the post-translocational GDP form in a manner similar to that of fusidic acid (3). However, in contrast to fusidic acid, which binds both EF-G and eEF2 and is a general translocation inhibitor, sordarin inhibits translation only in susceptible fungi, deriving its unique specificity from the source of eEF2 (3–5). The observation that eEF2 is the major determinant of sordarin specificity was confirmed by the identification of 15 unique sordarin-resistant alleles of EF-T1 and EF-T2 that encode eEF2 in S. cerevisiae. In our original characterization of 21 sordarin-resistant mutants, five mutations were not linked to the EF-T1 or EF-T2 genes. In this work, we show that these five mutations map to the essential ribosomal protein L10e.

The ribosome, although not contributing significantly to the fungal specificity of sordarin, is a critical partner in forming the stabilized post-translocational complex (3). Detection of a complex between fungal eEF2 and a labeled sordarin analog is strongly dependent upon the presence of ribosomes. L10, the prokaryotic counterpart of S. cerevisiae L10e, has been localized to the base of the stalk structure conserved in all large ribosomal subunits (6–8). The eukaryotic ribosomal stalk proteins L10e, L12eIA, L12eIIA, L12eIB, and L12eIIB in S. cerevisiae (9, 10) comprise a pentameric structure that is similar to the L10 and L7/L12 complex in Escherichia coli ribosomes. The conservation of the stalk structure has been visualized in recent cryoimages of both 70S (11), in which the binding position of the EF-G-GDP-fusidic acid complex is observed in detail, and 80S (12) ribosomes. The prokaryotic L7/L12 ribosomal proteins have been studied extensively by many physical and biological techniques, but much less is known about the structure and function of prokaryotic or eukaryotic L10. L10 is among the proteins reported to be cross-linked to eEF2 in 80S ribosomes by bifunctional reagents (13). In yeast, mutational analysis of the L10e ribosomal protein gene has shown that only L10e is essential, and that the carboxyl-terminal 132 amino acids of the L10e protein are required for viability. However, the L12e proteins that comprise the L10e/L12e pentameric complex are not essential (14). Several findings suggest that there are associations between the stalk proteins and elongation factors. Mutations in L7/L12 perturb both EF-Tu and EF-G functions in E. coli (15). Chemical cross-links have been observed between EF-Tu and EF-G and the L7/L12 complex (reviewed in Ref. 16). Of particular relevance to the present case, cross-links are observed between the EF-G and L7/L12 proteins in the presence of the nonhydrolyzable GTP analog GMPPCP, but not in the presence of GDP and fusidic acid (17, 18). L7/L12 proteins in the EF-G-fusidic acid-ribosome complex are resistant to trypsin proteolysis (19). Our current results add to the body of information implicating L10e and eEF2 interactions to be important in translocation. These studies define a role for a small region of L10e in mediating inhibition by a new class of natural product with unprecedented selectivity for fungal protein synthesis and provide evidence for a functional interaction between L10e and eEF2.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Compounds—Sordarin was isolated essentially as described from Sordaria arenosa (1), and preparation of


The sordarin analog L-793,422 has been described previously (3, 20). The sordarin-resistant strains sRb1, sRb2, sRb5, and sRb13, and sRb14 were generated essentially as described previously (3), except that mutant strains that demonstrated sordarin resistance linked to EFT1 or EFT2 were selected for characterization in this study. The wild-type strains harboring an episomal copy of either YCplac111 or YCpL12eIB, YCpL12eIIA, and YCpL12eIIB were verified by restriction analysis. Plasmid DNA pools of each construct described above were transformed into the E. coli DH5α (Life Technologies Inc.) strain DH5α, which was used for subcloning an episomal copy of the 5.7-kb HindIII fragment that encodes the sordarin target. The sordarin-resistant strains sRb1, sRb2, sRb5, and sRb13 were generated by introducing a 5.7-kb HindIII fragment into the E. coli DH5α (Life Technologies Inc.) strain DH5α, which was used for subcloning an episomal copy of the 5.7-kb HindIII fragment that encodes the sordarin target. The sordarin-resistant strains sRb1, sRb2, sRb5, and sRb13 were generated by introducing a 5.7-kb HindIII fragment into the E. coli DH5α (Life Technologies Inc.) strain DH5α, which was used for subcloning an episomal copy of the 5.7-kb HindIII fragment that encodes the sordarin target.

**Molecular Mapping of eEF2 Mutations—**Plasmid DNA from transformants with the resistance phenotype was sequenced with an ABI Prism 373 DNA Sequencer according to the manufacturer’s recommendations (Perkin-Elmer Applied Biosystems). Sequences were analyzed using Sequencer DNA analysis software (Gene Codes Corp.). The products of three additional independent PCR reactions were sequenced to rule out the possibility of errors due to polymerase infidelity.

**Cell Extracts—**Washed cells grown to logarithmic phase (A_{600}=1–1.5) in YPAD medium at 30 °C were disrupted, and post-ribosomal extracts were prepared essentially as described by Skogerson (22). Wild-type eEF2 was used as a final specific activity of 2.5 pmol/μg in the diphtheria-catalyzed ADP ribosylation assay. Ribosomes were prepared using standard procedures (23), and their concentration was estimated using the figure of 18.6 pmol/mg of S30 extract or at 0.39 pmol of [3H]L-793,422 bound with 0.92 pmol of purified eEF2, 3 pmol of salt-washed ribosomes, 0.1 μCi of [guanosine-8-3H]GTP (12 Ci/mmol), and increasing amounts of sordarin or analogs. After 30 min at 22 °C, 1 ml of ice-cold buffer was added, which followed by a final 1 ml wash. Under these conditions, approximately 0.1 mol of nucleotide was trapped per mol of eEF2 in the absence of any compound.

**GTase Assay—**Polymerization using S30 extracts was performed at 22 °C essentially as described by Hussain and Leibowitz (26) with poly(U) (Calbiochem) at 160 μg/ml as a template during the linear period of incorporation at 22 °C using [3H]phenylalanine (4000 cpm/ pmol) as a precursor. Polymerization reactions were performed with either intact ribosomes or those reconstituted from separated subunits and contained 0.05 A_{260} unit of ribosomes and 10 μg of S100 protein as a source of all soluble factors. In these assays, incorporation of [3H]phenylalanine was limited by the level of ribosomes.

**RESULTS AND DISCUSSION**

A Class of Sordarin-resistant Mutations Not Linked to eEF2—We have previously described S. cerevisiae mutants containing unique amino acid substitutions in eEF2 that confer resistance to sordarin (3). These mutants were obtained by spontaneous mutagenesis of eft1Δ/eft2Δ deletion strains harboring an episomal copy of either EFT1 or EFT2, genes that encode eEF2. Linkage of sordarin resistance to eEF2 was confirmed by demonstrating plasmid-dependent sordarin resistance in sensitive strains. In addition to the eEF2 mutants, five additional sordarin-resistant mutants (sRb1, sRb2, sRb5, sRb13, and sRb14) were identified that harbored wild-type episomal EFT1 or EFT2. All of these strains had sordarin IC_{50}s for growth inhibition in SC medium of 10–30 μg/ml (Fig. 1). No additional phenotypes were observed for these mutants in standard laboratory media, with the exception of a growth defect in strain sRb2 (data not shown).

To characterize the genetic elements that mediate sordarin resistance, diploids were generated from crosses of the sRb strains and wild-type strain YPH54. In growth inhibition assays, sRbYPH54 diploids exhibited a codominant phenotype at low concentrations of sordarin. Tetrad analysis of all diploids showed that sordarin resistance segregated 2s:2r, indicating that the resistance phenotype results from mutations in a single chromosomal gene (data not shown). Complementation testing was performed on sRbs sRb diploid strains to determine the number of genes represented. Approximately the same level of resistance to sordarin was observed among the sRbs sRb diploids, suggesting that the mutant strains belong to the same complementation group. This result was confirmed by tetrad analysis of the sRbs sRb mutant strains, indicating that the sRb mutation is defined by a single nuclear gene (data not shown).

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showed a marked reduction in sordarin sensitivity in in vitro translation assays using poly(U) as a template. This is illustrated for the sRb5 mutant in Fig. 2A. The other four mutants displayed essentially identical inhibition curves (data not shown), which shifted about 6-fold from the control value at low sordarin concentrations and showed no further inhibition at levels up to 5 μg/ml. This biphasic response could be due to the functional redundancy of a ribosomal protein(s) or to the limitations of the assay.

Fractionation of the S30 extracts into salt-washed ribosomes and S100, followed by reconstitution, did not alter the sordarin inhibition curve for the sRb mutants. However, experiments using S100 extracts and ribosomes from either the sordarin-sensitive parental strain or the sRb mutants allowed for the identification of the resistance determinant as the ribosome in all cases (sRb5 is shown in Fig. 2B). Ribosomes prepared from strain sRb5 were further separated into large and small subunits, and, as shown in Fig. 2C, resistance was found to be associated with the large ribosomal subunit via in vitro translation experiments. Reconstitution studies between subunits of wild-type strains and those of the other sRb strains gave similar results (data not shown).

Ribosomal Protein L10e Confers Sordarin Resistance—Identification of the large ribosomal subunit as the determinant of sordarin resistance, in addition to the biochemical and genetic associations of the stalk proteins with EF-G or eEF2 (11, 13, 15–19), focused our efforts on the ribosomal stalk proteins. Because both fusidic acid and sordarin stabilize the eEF2-GDP-ribosome complex, we hypothesized that proteins in the stalk were candidate biochemical targets of sordarin. In S. cerevisiae ribosomes, the stalk is composed of five proteins: L10e and a tetramer of L12eIA, L12eIIA, L12eIB, and L12eIIB. These proteins are the eukaryotic counterparts of the L10 and L7/L12 proteins in E. coli. S. cerevisiae L10e- and L12e-specific oligonucleotides were synthesized (Table I) and used in PCR amplification of genomic DNA from the sordarin-resistant strain sRb5. Putative L10e and L12e ribosomal protein genes were verified, subcloned, and transformed into sordarin-sensitive yeast. Colonies from individual transformations were pooled and plated on SC agar medium containing sordarin or tested in growth inhibition assays. After incubation for 4 days at 29 °C, colonies appeared only on plates with transformed cells containing episomal L10e from sRb5 (data not shown). Representative data from a growth inhibition assay are shown in Fig. 3A.

Based on these results, L10e clones from the four remaining sRb strains were generated and examined in a similar manner. As shown in Fig. 3B, L10e from all strains conferred sordarin resistance, although less for sRb2 than for the others, as was seen for the original isolated mutants (Fig. 1).

Plasmid DNA from the L10e transformed strains was recovered and sequenced. Several nucleotides that differ from the sequence published by Hunter Newton et al. (Ref. 10; GenBank™ accession number M26506) were identified (Fig. 4). To determine the specific change responsible for the resistant phenotype, the L10e gene from the parental strain YEFD12h was amplified by PCR and sequenced. A comparison of the published sequence with our parental strain showed that L10e in YEFD12h contains three nucleotide changes that result in three conservative amino acid substitutions: I4V, V27I, and
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Table I

Sequence of synthetic oligonucleotides used for PCR amplification of the S. cerevisiae L10e and L12e ribosomal protein genes

Sequences are written 5' to 3'; sense (+) and antisense (-) strands are indicated. Lower case letters represent sequences used for cloning the PCR products; upper case letters represent the partial DNA sequence from the L10e and L12e ribosomal protein genes (10) that were used for PCR amplification.

| L12e and L10e PCR cloning primers | Sense strand | Anti-sense strand |
|-----------------------------------|--------------|------------------|
| L12eIA + strand                   | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |
| L12eIA - strand                   | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |
| L12eIB + strand                   | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |
| L12eIB - strand                   | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |
| L12eIAA + strand                  | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |
| L12eIAA - strand                  | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |
| L12eIBB + strand                  | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |
| L12eIBB - strand                  | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |
| L10e + strand                     | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |
| L10e - strand                     | gcgcgcagatctCTGAGATGCAGATGACAGGCAGATCTGCCCACTTAC | gcgcgcagatctCCCAGAAGTGTTTTGAAGTTGAGTAGAAGG |

T281S. Based on this result, we were able to identify the L10e amino acid substitutions that confer sordarin resistance (Fig. 4). Amino acid substitutions selected from single nucleotide changes, with the exception of S134D, which results from the deletion of three nucleotides. All of the changes conferring resistance were found to be clustered within 10 residues located approximately in the middle of the L10e polypeptide.

**L-793,422 Binding Studies**—Fungal specificity by the sordarin class of compounds is conferred by the source of eEF2. However, for a stable interaction to be detected with [3H]L-793,422, ribosomes must be present (Table II). Titration of the ribosomes with a fixed amount of eEF2 showed that an approximately 3-fold excess of ribosomes was required to give saturation at about 0.85 pmol/pmol eEF2, with an apparent K_d of 2.5 nM. No substantial differences were seen for wild-type and wild-type eEF2. Also, the level of ribosomes required for saturation did not deviate significantly from the wild-type value for any of the mutants (data not shown).

The minimal requirements for the ribosomal contribution to binding were assessed by dissociating ribosomes into components (Table II). The L12e stalk was removed from ribosomes, i.e. core ribosomes, without significant binding loss. However, the separation of subunits caused a marked decrease in their ability to stimulate eEF2-associated binding. Residual binding in the presence of individual subunits was consistent with the approximately 10% cross-contamination of large subunits by light as estimated by Western blotting with L10e antiserum. Recombination of the separated subunits restored binding. Solubilized protein from complete ribosomes or large subunits did not stimulate the very low level of binding exhibited by eEF2 alone. Wild-type L10e purified by reverse phase chromatography also failed to stimulate eEF2 binding of sordarin (data not shown). Thus, the ribosomal component of binding requires both subunits and possibly the interface between them and cannot be supplied by soluble ribosomal proteins.

**Sordarin as a Translocation Inhibitor**—To examine translocation more directly, we assessed the stability of the nucleotide-eEF2-ribosome complex for each of the ribosomal mutants, using [guanosine-8-3H]GTP and a rapid filtration assay. The amount of labeled nucleotide trapped in the presence of increasing levels of sordarin is shown in Fig. 5A for wild-type eEF2 plus wild-type or mutant sRb5 ribosomes. Without the addition of sordarin, approximately 0.1 mol of nucleotide is trapped per mole of eEF2. Sordarin increased this to a maximum of 0.28 (± 0.007) mol for wild-type ribosomes (with an IC_50 of about 25 ng/ml), 0.23 (± 0.011) mol for sRb1 ribosomes, 0.18 (± 0.007) mol for sRb2 ribosomes, 0.20 (± 0.010) mol for sRb5 ribosomes, 0.22 (± 0.009) mol for sRb13 ribosomes, and 0.16 (± 0.005) mol for sRb14 ribosomes. Thus, the eEF2 complexes containing mutant ribosomes all showed a 20–40% reduction in sordarin-conferred stabilization when compared with wild-type ribosomal complex, consistent with their approximately equal resistance to sordarin for in vitro
Turnover rates in translocation are reflected in the ribosomal EF2-dependent GTPase activity. Fusidic acid at low millimolar levels strongly inhibits the ribosomal-dependent GTPase activity of yeast eEF2 (27). Sordarin, although exerting its effect with much higher affinity, has less effect on GTPase activity. Sordarin concentrations of $100 \text{ng/ml}$ maximally inhibited the GTPase activity of wild-type ribosomes and eEF2 by about 60%. Similar experiments with wild-type eEF2 and each of the ribosomal mutants showed similar titration curves (data not shown), but the inhibition of GTPase activity was reduced 30–40% relative to wild-type eEF2 and ribosomes. Fig. 5B shows a time course for sRb1 compared with wild-type ribosomes at a single saturating sordarin concentration of 1 $\text{mg/ml}$. Approximately the same reduction was obtained whether the mutant GTPase activity was lower than the wild-type (0.8 pmol/pmol eEF2/min), as for sRb1 (0.4 pmol/pmol/min eEF2), or higher (1.1 units for sRb2 and 1.2 units for sRb5). Consistent with these elevated GTPase rates, ribosomes sRb2 and sRb5 both showed somewhat elevated translation rates with wild-type eEF2 in the absence of sordarin under the established assay conditions (data for sRb5 may be seen in the legend of Fig. 2). Overall, the magnitude of the reduction in GTPase activity exhibited by the mutants resembles that seen for the nucleotide complex. Because we have demonstrated that the ribosomal mutants are not impaired in sordarin binding, the reduced sensitivity seen in GTPase and complex stability must represent a functional alteration in translocation for the mutants. The amino acid substitutions that result in the sordarin-resistant phenotype cluster to a 10-amino acid region approx.
approximately in the middle of the L10e polypeptide and suggest that this region may be important in conformational flexibility during translocation.

In our initial study, we emphasized the qualitative similarity between the action of sordarin and fusidic acid. Sordarin stabilizes the eEF2-ribosome-GDP complex in a manner similar to that of fusidic acid (Ref. 3 and this work). Quite compellingly, there is some cross-resistance between sordarin and fusidic acid resistance in eEF2 alleles; furthermore, sordarin-resistant mutations cluster in a pattern similar to that demonstrated for fusidic acid resistance alleles in *S. typhimurium* EF-G (28). The present data demonstrate the quantitative differences in the effects of sordarin and fusidic acid and suggest that upon closer analysis, their precise modes of action will most certainly differ.

Our results have implications for the physical and functional relationship between L10e and eEF2. Others have shown that L10 is exposed on the surface of 70S ribosomes in *E. coli* (29), and chemical cross-links have been observed between rat L10 and eEF2. Recently, a three-dimensional cryoelectron microscopy map of the ribosome-EF-G-GDP-fusidic acid complex has been obtained (11). In this structure, EF-G makes contacts with both the 30S and 50S subunits. Moreover, domains I and V of EF-G make contacts with the base of the L7/L12 stalk complex. Many reports have implicated the stalk complex in factor binding and translocation, and conformational changes in the stalk and EF-G are thought to drive the translocation process (6, 16, 29). The highly mobile nature of the stalk is well established, and a recent report proposes that conformational changes in the amino-terminal domains of L7/L12 are coupled with polypeptide synthesis (30). In the cryoelectron microscopy map of the ribosome-EF-G-GDP-fusidic acid complex, the stalk appears as a well-defined structure, presumably frozen in a stable conformation by fusidic acid. Sordarin and fusidic acid both stabilize the eEF2-ribosome-nucleotide complex in these mutants is impaired. Our genetic and biochemical analysis, their precise modes of action will most certainly differ.

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### Table II

**Binding of [3H]L-793,422 to ribosomal components**

| Components                  | [3H]L-793,422 bound |
|-----------------------------|--------------------|
| eEF2                       | 0.002              |
| eEF2 + ribosomes            | 0.156              |
| eEF2 + 60S subunit          | 0.016              |
| eEF2 + 40S subunit          | 0.005              |
| eEF2 + 6OS + 4OS subunits   | 0.145              |
| eEF2 + core ribosomes       | 0.161              |

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**Fig. 5.** A, mutant ribosomes show reduced sordarin-conferred stabilization of the eEF2-nucleotide complex. Binding of guanosine-8-[3H]GTP was performed with 0.92 pmol of wild-type eEF2 and 3 pmol of salt-washed ribosomes from wild-type (●) or sRB5 (○) on nitrocellulose filters as described under “Experimental Procedures.” B, mutant ribosomes show reduced stimulation of eEF2 GTPase activity. A time course of GDP release is shown as the number of moles released per mole of eEF2 for wild-type ribosomes (circles) and sRB1 ribosomes (squares) in the absence (open symbols) and presence (filled symbols) of 1 µM sordarin. The value without eEF2 (reaching approximately 0.2 mol at 10 min for both wild-type and sRB1 ribosomes) was subtracted in each case. GTPase activity for wild-type ribosomes in the presence of 2 mM fusidic acid is shown (×). Data shown are the averaged results of a single experiment performed in duplicate reactions with duplicate samples.
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