Elongation Factor 2 as a Novel Target for Selective Inhibition of Fungal Protein Synthesis*

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Elongation factor 2 (EF2) is an essential protein catalyzing ribosomal translocation during protein synthesis and is highly conserved in all eukaryotes. It is largely interchangeable in translation systems reconstituted from such divergent organisms as human, wheat, and fungi. We have identified the sordarins as selective inhibitors of fungal protein synthesis acting via a specific interaction with EF2 despite the high degree of amino acid sequence homology exhibited by EF2s from various eukaryotes. In vitro reconstitution assays using purified components from human, yeast, and plant cells demonstrate that sordarin sensitivity is dependent on fungal EF2. Genetic analysis of sordarin-resistant mutants of Saccharomyces cerevisiae shows that resistance to the inhibitor is linked to the genes EFT1 and EFT2 that encode EF2. Sordarin blocks ribosomal translocation by stabilizing the fungal EF2-ribosome complex in a manner similar to that of fusidic acid. The fungal specificity of the sordarins, along with a detailed understanding of its mechanism of action, make EF2 an attractive antifungal target. These findings are of particular significance due to the need for new antifungal agents.

Sordarin, was described as an antifungal agent in 1970 (2, 3), but the mode of action of this family has not been examined until now. In this report, we show that sordarins specifically bind to the S. cerevisiae EF2-ribosome complex and block protein synthesis by inhibiting the release of EF2 from the post-translational ribosome. Our observations show that it is possible to inhibit fungal EF2 specifically, which may provide an opportunity for developing antifungal agents with a unique and selective mechanism of action.

EXPERIMENTAL PROCEDURES

Sordarin was isolated essentially as described for Sordaria armenosa (2). Reticulocyte and wheat germ lysates were obtained from Promega. Assays—IC₅₀ values were determined from growth inhibition assays in which cells were inoculated at 2 × 10⁵ cells/ml in TPAD medium (4) containing sordarin serially diluted 2-fold from 0.5–100 μg/ml, followed by incubation at 29 °C for approximately 16 h. S30 extracts and translation factors EF1, EF2, and EF3 were prepared from S. cerevisiae cells harvested in mid-to-late logarithmic phase (A₅₀₀–2). Translation assays using S30 extracts were performed at 22 °C essentially as described in Ref. 5 with poly(U) (Sigma) at 160 μg/ml as message and [3H]phenylalanine as precursor during the linear period of incorporation. Translation factors were purified essentially as described by Skogerson (6) except for the substitution of a Mono Q cartridge (Pharmacia Biotech Inc.) for DEAE Sephadex. Yeast EF2 was nearly homogeneous by SDS-gel electrophoresis and silver staining and had a final specific activity in the diphtheria catalyzed ADP-ribosylation assay (6) of 2.5 pmol/μg. Rat liver EF1 and EF2 were prepared as described in (7) and the wheat germ proteins as in Ref. 8. Ribosomes were prepared from all three sources by sedimentation three times through 0.5 M KCl, 20% sucrose, 10 mM MgCl₂ cushions and concentrations estimated using the figure of 18.6 pmol/A₅₀₀ unit. In vitro translation assays with purified ribosomes and translation factors were performed as described for S. cerevisiae (7) except that [3H]Phe-tRNA was prepared as precursors at a specific activity of 2000 dpm/μg.

[H]IL-793,422 (Fig. 1) was prepared at 20 μCi/μg (8000 Ci/mmol) and a concentration of 0.004 mg/ml by the Drug Metabolism Department at Merck Research Laboratories. Each binding assay contained 2 μg of yeast S-30, 25 μg GTP-S3 and Buffer A (50 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 1 mM EDTA) and 0.4 ng of [3H]IL-793,422 in a volume of 100 μl in a Microfuge tube. After 20-min incubation at room temperature, the micro tube volume was applied to a minicolumn (Isolab GS-QS quick-sep) packed with 1.6 ml (settled volume) of Sephadex G-75 (Type 120 from Sigma) presoaked in Buffer B (Buffer A plus 150 mM NaCl). As soon as the sample entered the gel bed, 0.7 ml of Buffer B was added and eluate collected in minivalves for counting.

Yeast Strains and Plasmids—The sordarin-resistant strains sR1 and sR2 were generated by selecting spontaneous mutants of the parental strain YPH88 (MATa ade2 leu2 lys2 trp1 ura3) (9) on SC medium (4) containing 5 μg/ml sordarin. The haploid S. cerevisiae strain YEFD12b/pURAS-EFT1 (MATa ade2 leu2 his3 trpl1 trp1 HS1:His3 ef2/ΔTRP1), deleted for the genomic copies of EFT1 and EFT2, and harboring the plasmid pURAS-EFT1 for viability, has been described (10). Strain YEFD12b/pURAS-EFT1 was used for transformation with pUC21, followed by expression of the plasmid pURAS-EFT1. Strains deleted for either EFT1 or EFT2 were constructed by mating YEFD12b/pURAS-EFT3 with the strain YPH54 (MATa ade2 his3 trp1 ura3) (9) to obtain spores with the genotype EFT1/Δ TRP1 or EFT2/Δ HIS3. The EFT2 yeast expression plasmid YCpEFT2 was constructed by subcloning a 5-kilobase pair BamHI-PstI fragment of DNA that includes the native promoter and the entire E2 coding region into plasmid YEp111 (11).

Molecular Mapping of E2 Mutations—Plasmid-dependent sordarin-resistant mutants were spontaneously generated from the eft1Δ/eft2Δ deletion strain YEFD12b harboring an episcopal copy of EFT1

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1 The abbreviations used are: GTP, guanosine 5’-3’-O-(thiotriphosphate); PCR, polymerase chain reaction.

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EF2 and Inhibition of Fungal Protein Synthesis

Sordarin as a Selective Inhibitor of Fungal Protein Synthesis—Exposure of intact S. cerevisiae to low concentrations of sordarin rapidly inhibits the incorporation of \(^{3}H\)threonine into proteins while having no effect on incorporation of uracil into RNA or adenine into DNA.\(^2\) The IC\(_{50}\) for this effect (6 \(\mu\)g/ml) corresponds closely to the IC\(_{50}\) values for growth inhibition of wild-type strains in YPAD medium. In contrast, sordarin has no effect on HeLa protein synthesis (leucine incorporation) or cell proliferation at concentrations of 100 \(\mu\)g/ml.\(^2\) In vitro translation experiments using S30 extracts of S. cerevisiae and a poly(U) message revealed that sordarin inhibits phenylalanine incorporation (IC\(_{50}\) \(\approx 30\) ng/ml) while having no effect on similar systems from rabbit reticulocyte or wheat germ at levels of up to 100 \(\mu\)g/ml.\(^2\) Based on these findings it appears that sordarin is a specific inhibitor of protein synthesis in fungi while having no effect on other eukaryotic systems, a specificity within eukaryotes that is without precedent for known translation inhibitors.

Sordarin Binding Assay—Hydrolytic cleavage of sordarin to sordarose and sordaricin abolishes activity both for inhibition of whole cell growth and for in vitro translation. However activity is restored by replacement of the sugar with a short chain alky1 ether. L-793,422 (Fig. 1A), a sordaricin derivative with an isobutyl ether side chain, is 1000-fold shorter than the parent compound. L-793,422 (Fig. 1A), a sordaricin derivative with an isobutyl ether side chain, is 1000-fold shorter than the parent compound. L-793,422 (Fig. 1A), a sordaricin derivative with an isobutyl ether side chain, is 1000-fold shorter than the parent compound. L-793,422 (Fig. 1A), a sordaricin derivative with an isobutyl ether side chain, is 1000-fold shorter than the parent compound.

RESULTS AND DISCUSSION

Fungal Specificity Conferred by EF2—The ability of EF2 and ribosomes purified from rat liver and from wheat germ to bind \(^{3}H\)L793,422 in the presence of GTP was examined. In contrast to S. cerevisiae EF2, neither rat liver nor wheat germ EF2 shows any binding when tested with ribosomes from rat liver, wheat germ, or yeast. Nor does binding occur using S. cerevisiae ribosomes and either higher eukaryotic EF2. However, substantial binding is conferred by S. cerevisiae EF2 upon addition of ribosomes from either rat liver or wheat germ (Fig. 1A). Thus EF2 is the determinant of the observed fungal specificity of sordarin. Furthermore, fungal specificity in polymerization can be demonstrated in reconstituted translation assays dependent on added purified EF2. Polyphenylalanine formation from \(^{3}H\)Phe-tRNA as precursor by rat liver ribosomes plus rat liver EF1 is insensitive to sordarin when rat liver EF2 is used, but sensitive with yeast EF2. Incorporation by yeast ribosomes plus yeast EF1 and EF3 is sensitive with yeast EF2, reconstituting the sensitivity of the unfractionated system, but becomes insensitive when rat liver EF2 is used (Fig. 1B).

Stabilization of the EF2-Ribosome Complex—During the translocation cycle, GTP is bound by the EF2-ribosome complex, followed by an extremely rapid hydrolysis to EF2-GDP, and a conformational change that releases EF2 for the next round of translocation (13). Fusidic acid is a universal EFG and EF2 inhibitor that inhibits translocation by stabilizing the EF2-GDP-ribosome complex (14). Fusidic acid stabilization was demonstrated for the S. cerevisiae complex by measuring cold excess GTP exchange with prebound ring-labeled \(^{3}H\)GTP.

Fig. 1. Specificity is conferred by EF2. A, binding of \(^{3}H\)L793,422 by 0.5 pmol of purified EF2 from the sources noted on the \(x\) axis and 3.5 pmol of salt-washed ribosomes from rat liver (solid bar), wheat germ (stippled bar), and yeast (open bar). B, polymerization with purified components. Solid circles, rat liver ribosomes plus rat liver EF1 and rat liver EF2; open squares, rat liver ribosomes plus rat liver EF1 and yeast EF2; open circles, yeast ribosomes plus yeast EF1, yeast EF2, and yeast EF3; filled squares, yeast ribosomes plus yeast EF1, rat liver EF2, and yeast EF3. When present, as noted for each combination, components were used in the following quantities in 50-\(\mu\)l reactions with 5 pmol of \(^{3}H\)Phe-tRNA: rat or yeast salt-washed ribosomes (0.05 \(A_{260}\) units); rat EF1 (1 \(\mu\)g of partially purified, free of EF2) and yeast EF1 (0.4 \(\mu\)g, free of both EF2 and EF3); rat EF2 (0.2 pmol) or yeast EF2 (0.15 pmol); yeast EF3 (0.2 \(\mu\)g). Incorporation for 5 min (22 °C) was still linearly time-dependent and was approximately 2 pmol in each uninhibited system. Results are expressed as percentage of incorporation in the absence of sordarin for each combination.

Sequestration chromatography (6), the soluble binding component comigrates with EF2.

\(^2\) J. Nielsen, unpublished results.
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Fig. 2. Stabilization of EF2-nucleotide-ribosome complex by sordarin as measured at 22°C by displacement of prebound [guanosine-8-3H]GTP (12 Ci/mmol) by 1000 × GTP added to a binding assay containing 0.5 pmol of EF2 and 3.5 pmol of ribosomes, both from S. cerevisiae, at t₀. Gel filtration was initiated at the times noted. Squares, no addition; circles, 1 mM fusidic acid; diamonds, 20 μg/ml sordarin.

Fig. 3. The amino acid sequences of EF2 and S. typhimurium EFG were aligned using the GAP alignment tool from the Genetics Computer Group, Version 7, April 1991, 575 Science Drive, Madison, WI. Regions of homology between the EF2 and EFG proteins are illustrated by solid bars; gaps in homology are depicted by broken bars. EFG domains are indicated (16, 17). Substitutions that result in fusidic acid resistance in EFG, and sordarin resistance in EF2, are indicated by arrows. The scale at the bottom of the figure is relative only to the aligned sequences of EFG and EF2.

Table I

| A. Plasmid Mutation | Sordarin IC₅₀ (μg/ml) | Fusidic acid IC₅₀ (μg/ml) |
|---------------------|-----------------------|--------------------------|
| EFT1 (Wild-type)    | 0.5                   | 3                        |
| EFT2 (Wild-type)    | 1                     | 3                        |
| pSR4                | Y521 → S              | 35                       | 6                        |
| pSR5                | Y521 → D              | 60                       | 20                       |
| pSR6                | I529 → T              | 30                       | 3                        |
| pSR7                | G790A                 | >100                     | No polymerization        |
| pSR8                | G490 → E              | 45                       | 3                        |
| pSR9                | F559 → L              | >100                     | >200                     |
| pSR11               | P559 → R              | >100                     | 100                      |
| pSR12               | P727 → S              | >100                     | 3                        |
| pSR13               | R180 → G              | 15                       | >200                     |
| pSR15               | S523 → F              | >100                     | >200                     |
| pSR16               | Y521 → N              | 20                       | 60                       |
| pSR17               | V774 → F              | >100                     | 3                        |
| pSR20               | V187 → F              | 20                       | 10                       |
| pSR26               | A562 → P              | >100                     | 15                       |
| pSR27               | S523 → F              | >100                     | 20                       |

B. Genomic mutations

| sR1 | A562→P | >100 | 15 |
| sR2 | V187→F | 15   | 200 |

Summary of EF2 Mutations

Episomal (A) and genomic (B) sordarin resistant mutants were generated and identified as described under “Experimental Procedures.” IC₅₀ values were determined from growth inhibition assays in which cells were inoculated in rich medium containing sordarin serially diluted 2-fold from 0.2–100 μg/ml, followed by incubation at 29°C for approximately 16 h. In vitro translation assays for fusidic acid sensitivity were performed with S30 extracts prepared from each mutant, poly(U) at 160 μg/ml and [3H]phenylalanine (4000 dpm/μmol) as precursor, using ionic and cofactor conditions described in Ref. 6.

Genetic Linkage of Sordarin Resistance—Genetic confirmation that fungal EF2 confers specificity to sordarin was demonstrated by analysis of mutant yeast strains resistant to sordarin. Spontaneous mutants resistant to sordarin were selected on SC medium containing 5 μg/ml sordarin; these appeared at a frequency of approximately 1 × 10⁻⁹. Progeny from crosses of the sordarin-resistant strains sR1 and sR2 with strain YEFT2ΔΔtrp1 (eft2ΔΔTRP1) that is deleted for the genomic copy of the EFT2 gene were analyzed. Dissection of 20 tetrad from each cross showed that sordarin resistance was never associated with tryptophan prototrophy, confirming genetic linkage, and that a mutant allele of eft2 is responsible for sordarin resistance in both sR1 and sR2 strains.

Identification of Mutations That Confer Sordarin Resistance—To facilitate determining which substitutions can result in sordarin resistance, mutants conferring plasmid-dependent resistance were spontaneously generated by plating cells on solid medium containing sordarin, using eft1ΔΔ/eft2ΔΔ deletion strains that harbor an episomal copy of EFT1 or EFT2. Fifteen mutant strains were chosen that range in resistance from having an IC₅₀ = 10 μg/ml sordarin, to an IC₅₀ = 100 μg/ml (Table I). Plasmids conferring sordarin resistance were recovered, cloned, purified, and sequenced. A single base change causing an amino acid substitution was identified in each clone, with the exception of pSR7, which has a three base pair deletion, resulting in the loss of G790 (Table I). Based on the locations of the mutations in the episomal copies of the EFT1 and EFT2 genes, we used PCR to amplify specific regions of EFT2 from genomic DNA prepared from the original sR1 and sR2 mutants. Single base changes that result in amino acid substitutions in each mutant were identified. The amino acid changes confering resistance to sordarin are clustered into three regions of the EF2 protein (Fig. 3). Amino acid sequence alignment of EF2 with its prokaryotic counterpart EFG demonstrates that the EF2 substitutions are located in regions with homology to domains I, III, and IV in EFG. These domains of EFG are thought to interact with one another during GTP hydrolysis and translocation (15–17). The sequence alignment also revealed that mutations in EF2 conferring resistance to sordarin are located in proximity to mutations in EFG that give rise to fusidic acid resistance (18, 19) (Fig. 3). Since fusidic acid does not permeate yeast cells, translational sensitivity to fusidic acid was determined in S30 extracts prepared from each sordarin-resistant mutant. Many mutations that confer resistance to sordarin also confer resistance to fusidic acid (Table I), although levels of resistance to the two drugs are not closely correlated. The partial cross resistance of mutations and the proximity of mutations that confer sordarin and fusidic acid resistance in the alignment of EF2 with EFG support the biochemical evidence of mechanistic similarity of the two drugs. One or more of the EF2 residues altered in the resistant mutants may be located in the sordarin binding site, but there is not sufficient evidence, without cross-linking or other biochemical approaches, and a three-dimensional structure for EF2, to identify the binding site.

Sordarin belongs to a class of natural products with multiple
functional groups readily accessible to chemical modification. The addition of this class to the small number of known EF2 inhibitors (fusidic acid family, ADP-ribosylators, both universal for eukaryotic translocation) provides a powerful new tool for dissecting the EF2 and ribosomal sites involved in translocation. Furthermore, the ability to inhibit protein synthesis specifically in fungi with sordarin demonstrates the potential for exploiting EF2 as a target for developing novel antifungal agents at a time when resistance to current agents is increasing. These findings show that homology between essential proteins and processes in the host and pathogen does not necessarily exclude them as potential targets for selective chemotherapy.

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