Ribosomal P-protein Stalk Function Is Targeted by Sordarin Antifungals*

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Sordarin derivatives are remarkably selective inhibitors of fungal protein synthesis. Available evidence points to a binding site for these inhibitors on elongation factor 2, but high affinity binding requires the presence of ribosomes. The gene mutated in one of the two isolated complementation groups of Saccharomyces cerevisiae mutants resistant to the sordarin derivative GM193663 has now been identified. It is RPP0, encoding the essential protein of the large ribosomal subunit stalk rpP0. Resistant mutants are found to retain most of the binding capacity for the drug, indicating that mutations in rpP0 endow the ribosome with the capacity to perform translation elongation in the presence of the inhibitor. Other proteins of the ribosomal stalk influence the expression of resistance, pointing to a wealth of interactions between stalk components and elongation factors. The involvement of multiple elements of the translation machinery in the mode of action of sordarin antifungals may explain the large selectivity of these compounds, even though the individual target components are highly conserved proteins.

The ribosomal stalk is a defined morphological feature most evident in prokaryotic ribosomes, where it is composed of the L7/L12 tetramer, L10 and L11 proteins, and a highly conserved domain of the 23 S rRNA termed the “GTPase center.” The stalk plays a prominent role in the elongation phase of ribosomal protein synthesis (see Refs. 1 and 2 for a review).

Equivalent structures have been visualized on eukaryotic ribosomes (3) and functional homologies with the bacterial components have been described (see Ref. 4 for a review). The recent nomenclature proposal for Saccharomyces cerevisiae ribosomal proteins (5) will be followed throughout this manuscript. In this organism, proteins rpP1α, rpP1β, rpP2α, and rpP2β take the place of the bacterial L7/L12 tetramer. rpP0 corresponds functionally to bacterial L10 and rpL12 to bacterial L11 (6–10). rpP0 is thought to play a central role in stalk architecture, interacting with rpL12 and 26 S rRNA at its amino-terminal half and with the other four P-proteins at its carboxyl-terminal end (4, 11). None of the P1/P2 proteins are absolutely essential for growth, although in the absence of at least one member of each class (P1 and P2), vegetative growth is strongly retarded (12).

The molecular detail of the interplay between stalk components and elongation factors is unknown. Recent morphological evidence (13) indicates that elongation factors make multiple contacts with stalk components, and a detailed footprinting analysis of EF-G on rRNA place the factor at the base of the stalk (14). Resolution is however not high enough to ascribe roles to individual molecules.

Semisynthetic derivatives of the natural product sordarin (15, 16) have proven to be highly potent inhibitors of eukaryotic protein synthesis, with remarkable selectivity for the fungal translation machinery (17, 18). Sordarin inhibitors seem to bind to elongation factor 22,1 (18), and point mutations on the factor make cells resistant to the inhibitors3 (18), but high affinity binding requires the presence of ribosomes,4 and resistant mutants were detected, which did not map on EF2.

This work describes the gene mutated in a second complementation group of S. cerevisiae mutants resistant to GM193663, a potent sordarin derivative. The gene encodes the ribosomal stalk protein rpP0. It is further shown that other stalk components are also involved in the translation elongation function inhibited by GM193663, substantiating the ribosomal requirement for binding of the inhibitor to EF2 and pointing at interactions between the factor and specific stalk components.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions—**All the strains used in this study are derivatives of S. cerevisiae SEY6210 (Mat α ura3–52 leu2–3, 112 his3Δ200 trp1Δ901 lys2–81 suc2–52, its ade2–101 derivative SEY6211 (S. Emr), S. cerevisiae 373 (Mat a ade2–101) (A. Jimenez), S. cerevisiae W303 (Mat a leu2–3, 112 trp1·1 his·3–11, 15 ade 2-1 can 1–100) and S. cerevisiae W303ΔGp0 (W303 strain with the cassette pGalI-rP0-URA3 replacing the endogenous RPP0 locus) (19). For genetic mapping of the FPR2–15 mutation, crosses were made between strain W303ΔGp0 and W303 derivatives D46 (rpp1b::TRP1 rpp2a::URA3) and D57 (rpp1a::LEU2 rpp2b::HIS3). Heterologous rpP0 proteins were expressed in the W303ΔGp0 strain growing in glucose, to repress the chromosomal RPP0 gene, using the centromeric plasmid pFL39-HIS and the endogenous S. cerevisiae promoter. All W303 derivatives and pFL constructs were obtained from the laboratory of J. P. G. Ballesta (12, 19).

YPD (0.67% yeast nitrogen base without amino acids, 2% glucose, and auxotrophic supplements as required) and SD (1% yeast extract, 2% peptone, 2% glucose) media, methods for genetics crosses, sporulation of diploids, and dissection of ascii have been described previously (20). Yeast transformations were done by the lithium acetate procedure (21). Spontaneous resistant mutants were selected on YPD medium plates with 100 μg/ml sordarin derivative GM193663. P-protein gene disruptions on strains 6210, FPR1–19, and FPR2–15 were carried out according to standard procedures by allele replacement, using different constructions for genes rpp1a, rpp1b, rpp2a, and rpp2b, all obtained from the laboratory of J. P. G. Ballesta (12). Gene disruptions were checked by PCR3 or Southern blots (data not shown).

**In Vitro Activity and Binding Assays—**Growth inhibitory activity of GM193663 was determined on liquid medium by the antibiotic 2-fold serial dilution technique; 100 μl of YPD were inoculated with 104 colony-forming units/well. IC50 was defined as the minimal concentration of compound that inhibited 50% of the control growth after 24 h of incuba-
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Results

Mutations in Ribosomal Protein P0 Confer Resistance against Sordarin Antifungals—It has been communicated previously that genetic analysis of spontaneous mutants resistant to the sordarin antifungal GM193663, an inhibitor of translation elongation, identified two different genetic complementation groups, denominated FPR1 and FPR2. The major one, FPR1, grouped mutations in EF2, encoding translation elongation factor 2. FPR2 was composed of just four mutants and, contrary to expectations, did not arise from EF72, the second EF2, encoding EF21 in S. cerevisiae. Growth and resistance characteristics of the mutants in rich medium are presented in Table I. All mutants displayed cross-resistance on plates to other members of the sordarin family, but not to the protein synthesis inhibitors cycloheximide, hygromycin, or verrucarin A, showing that sordarins act by a mechanism different from the one used by those other inhibitors.

Protein synthesis in a cell-free extract from an FPR2 mutant also displayed a reduced sensitivity toward GM193663 inhibition, thereby ruling out a transport mechanism of resistance. To investigate the identity of the gene mutated in the FPR2 group, an educated guess was made considering that the most likely possibilities were genes encoding macromolecules that functionally interacted with EF2. Candidates included proteins of the large ribosomal subunit stalk, namely rpP0, the acidic P-proteins, and the rpL12 protein (formerly rpL15) located in the GTPase center (6–8, 10, 23). rRNA was an unlikely candidate given the semidominant nature of the mutations and the large number of rRNA genes present in the cell. Strains genetically tagged at the loci encoding stalk proteins rpP0, rpP1, rpP2, and rpP2β were obtained from the laboratory of J. P. G. Ballesta (12) and crossed to GM193663R mutant FPR2–15, which displayed the most dominant resistance phenotype. Tetrad analysis did not detect a genetic linkage between the resistance locus and the P-proteins, except in the case of rpP0, where a tight genetic linkage between the resistance mutation and RPP0, the rpP0 locus, was found. All 36 tetrads dissected displayed the parental diatype phenotype. This result strongly suggested that the resistance and RPP0 loci were very close to each other or were the same one.

RPP0 was amplified and cloned from the four mutants in group 2 as well as from their corresponding parental strains. The cloned genes were transformed into a sensitive haploid strain, and the transformants were tested for their sensitivity to GM193663. Strains transformed with an RPP0 gene from a resistant strain became resistant themselves in all cases, confirming that the gene mutated in the second complementation group was RPP0. Thus, mutations in protein rpP0, an essential protein of the large ribosomal subunit, can confer a level of resistance to sordarins higher than the one afforded by mutations in EF2, the proposed binding protein for sordarins (Table II). This could be the first such occurrence described, and it strongly suggests that rpP0 and EF2 interact functionally.

Resistance Mutations Cluster Together in the rpP0 Sequence—Chromosomal RPP0 genes from the four mutants were cloned by gap repair and analyzed by single-stranded conformational polymorphism (see “Experimental Procedures”). Mobility changes were found in the central one-third of all alleles. The region was sequenced on both strands, and amino acid changes were detected in all mutants (Table I, Fig. 1). To confirm that the observed changes were not introduced by the PCR, genomic fragments from the four mutants were recovered by the plasmid gap repair technique (24). A wild type RPP0 gene was gapped by removing all the open reading frame
except the last 86 amino acids and then transformed into the resistant mutants. Plasmids were recovered from stable transformants, sequenced, and re-transformed into a wild type strain. All recovered plasmids had the expected mutation and conferred resistance to GM193663, establishing that the observed amino acid changes (Table I) were responsible for the resistance to the inhibitor. Unexpectedly, spontaneous mutant FPR2–17 had four nucleotides changed, leading to two consecutive substitutions in the amino acid sequence of the protein.

Comparison of the sequence of the rp0 region, where the GM19366339 mutations were found across different phyla (Fig. 1), showed that this domain is a highly conserved one and hence presumably important for the function of rp0. It is thus somewhat surprising that the mutations do not impede the normal function of the protein, as indicated by the nearly wild type growth rate of the mutant strains in the absence of the inhibitor (Table I). In fact, there seems to be a lot of flexibility in the primary sequence requirements for rp0 function. The group of J. P. G. Ballesta has shown that the yeast ribosome can function with P0 proteins from different species.4 Using their strains, we have found that GM193663 has a higher IC50 for growth inhibition in S. cerevisiae cells carrying rp0 proteins from Dictostelium or rat than in cells expressing wild type rp0 (Table III). This confirms that rp0 plays a role in the mode of action of sordarin compounds and shows that it is at least partly responsible for the selectivity of these inhibitors.

Mutations in rp0 Do Not Abolish the Binding of the Inhibitor to Macromolecules—Sordarin is a close analogue of GM193663 (Fig. 2) and both compounds display cross-resistance and compete for binding to macromolecules.2 Fig. 2 shows [3H]sordarin binding to macromolecules in the rp0 mutants. Substantial binding was found in all mutant strains, showing that growth, and therefore protein synthesis, can take place in the presence of nearly wild type levels of inhibitor binding in some strains. Data from an EF2 mutant have been included for comparison, and it can be seen that in this case resistance is accompanied by the total loss of sordarin binding capacity, as described previously1 (Fig. 2). The measured binding is to the ribosomal fraction of the extracts plus associated cytoplasmic factors, because the post-ribosomal supernatant showed negligible binding (data not shown). Therefore, the mechanism of resistance mediated by the rp0 protein is different from the simple loss of affinity for the drug, and it may be related to the molecular details of the interaction between EF2 and the ribosomal stalk.

Other Proteins of the Ribosomal Stalk Modulate Resistance Conferred by rp0—Given the known or inferred interactions between the acidic P-proteins and rp0 (see Ref. 4 for a review), it was of interest to know if lack of the former affected resistance due to rp0, which would substantiate the relevance of the interactions between P-proteins in translation elongation. All four nonessential P-proteins were interrupted singly and in pairs in the FPR2–15 mutant background. It was found that deleting either rpP1a or rpP2b reduced resistance levels more than 10-fold. Deletion of either rpP1b or rpP2a did not have a significant effect. Deleting both rpP1a and rpP2b had an additive effect, whereas deleting the other pair did not affect resistance (Table II). The effect of the protein composition of the ribosomal stalk on the sensitivity toward GM193663 is observed in the mutant RPP0 background only. The same deletions do not affect the sensitivity of a wild type strain (data not shown). These results confirm that the four P-proteins are not functionally equivalent, with rpP1a and rpP2b establishing a

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4 M. A. Rodríguez-Gabriel, M. Remacha, and J. P. G. Ballesta, submitted for publication.
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The influence of the stalk on the sensitivity toward sordarin antifungals seems to be specific for this type of translational inhibitors, because deleting the acidic P-proteins, either on a wild type strain or in the FPR2−15 mutant, did not change their sensitivity toward a different elongation inhibitor, cycloheximide. However, GM193663 resistance mediated by a mutant EF2 was also diminished by deleting rpP1α and to a lesser extent by deleting rpP2β (Table II). This is in agreement with the findings presented above and further indicates that the soluble factor establishes functional interactions with the ribosomal stalk, and not only with rpP0, but also with rpP1α and perhaps with rpP2β as well.

**DISCUSSION**

Sordarins are a new family of antifungal compounds and, together with tenuazonic acid (25), the only protein synthesis inhibitors reported to show selectivity between different eukaryotes (17, 18). They have been described to target and bind to elongation factor 21,2 (18), but it was also immediately apparent that the inhibitor's mode of action involved additional cellular components, because resistant mutants were obtained outside EF2, and high affinity binding of the drug to macromolecules required the presence of ribosomes, suggesting that the binding site on EF2 is fully formed only after the factor interacts with a ribosome. In this work, experiments are described showing that rpP0, an essential component of the ribosomal large subunit stalk, plays a large role in determining the sensitivity to sordarin antifungal GM193663, as single point mutations in rpP0, and substitution of heterologous rpP0 proteins for the endogenous one, reduce measurably the sensitivity of yeast toward these compounds (Tables II and III). The results also indicate that there is a functional interaction between rpP0 and EF2, because mutations in any one of them render cells insensitive to the same translational inhibitor. This fits well with evidence indicating that elongation factors are probably recruited to the ribosome through interactions with the stalk (7, 9, 13, 26).

The mechanism of resistance afforded by mutations in rpP0 is intriguing, and its elucidation may shed light into the molecular details of ribosomal translocation. The binding capacity for the inhibitor has been retained to a large extent by the resistant mutants (Fig. 2). If the hypothesis that sordarin antifungals impede conformational changes in EF2 is correct1 (18), then changes in rpP0 could somehow force or facilitate the appropriate bending of the EF2 molecule. According to the data presented here, in order for rpP0 to be able to “help” EF2 when blocked by GM193663, the protein must be part of a ribosomal stalk in which rpP1α and rpP2β are present. Stalk structures deficient in either of those proteins substantially reduce the capacity of mutant rpP0 to overcome the presence of the inhibitor. Interestingly, rpP1α and rpP2β are also the P-proteins whose deletion has the largest effect on growth rate (12). rpP1α may be able to establish some interaction with EF2 by itself, because lack of this ribosomal protein also reduces significantly resistance levels afforded by mutations in EF2 (Table II). There seems to be also an effect of the rpP2β deletion on EF2-mediated resistance, although the reduction in the IC50 of the inhibitor is smaller in this case. All this hints at a wealth of functional interactions between elongation factors, or at least EF2, and components of the ribosomal P-protein stalk. Pairwise physical interactions between all these proteins are currently being analyzed by genetic and biochemical means.

The screen for GM193663R mutants is by no means saturated. Ongoing work at several laboratories will possibly detect additional gene products involved in the mode of action of sordarin antifungals, which may in turn uncover new functional interactions, or confirm suspected ones, between components of the translation apparatus.

In yeast there are described ribosomal protein mutations involved in resistance to cycloheximide, cryptopleurine, trichloroderm, and now sordarin analogues (27–31). Sordarin antifungals may however be unique in targeting the functional interaction between a soluble translation factor and ribosomal proteins, in such a manner as to allow both types of proteins to influence the cell's sensitivity to the inhibitor. The contribution of multiple elements to sensitivity may explain the large selectivity of these compounds, despite the high sequence conservation between the individual components of the eukaryotic translation apparatus. Sordarin antifungals are very selective drugs with a good therapeutic potential, and they are also turning out to be useful tools for the analysis of the elongation cycle during protein translation.

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**REFERENCES**

1. Liljas, A. (1990) in *The Ribosome: Structure, Function and Evolution* (Hill, W. E., Dahlberg, A., Garreti, R. A., Moore, P. B., Schlessinger, D., and Warner, J. R., eds) pp. 309–317, American Society for Microbiology, Washington, D. C.
2. Gudkov, A. T. (1997) FERS Lett. 407, 253–256
3. Verschoor, A., Srivastava, S., Grassucci, R., and Frank, J. (1996) *J. Cell Biol.* 133, 495–505
4. Ballesta, J. P. G., and Remacha, M. (1996) Prog. Nucleic Acid Res. Mol. Biol. 55, 157–188
5. Mayer, W. H., Planta, R. J., Ballesta, J. P. G., Lee, J. C., Mizuta, K., Suzuki, K., Warner, J. R., and Woolfard, J. (1997) *Nucleic Acids Res.* 25, 4872–4875
6. Uchiumi, T., Kikuchi, M., Terao, K., Iwasaki, K., and Ogata, K. (1986) *Eur. J. Biochem.* 156, 37–48
7. Uchiumi, T., Tsumura, T., and Kominami, R. (1990) *J. Biol. Chem.* 265, 89–95
8. Uchiumi, T., and Kominami, R. (1992) *J. Biol. Chem.* 267, 19179–19185
9. Uchiumi, T., and Kominami, R. (1994) *EMBO J.* 13, 3389–3394
10. Uchiumi, T., and Kominami, R. (1995) *J. Biol. Chem.* 272, 3392–3398
11. Santos, C., and Ballesta, J. P. G. (1995) *J. Biol. Chem.* 270, 20608–20614
12. Remacha, M., Santos, C., Bermejo, B., Naranda, T., and Ballesta, J. P. G. (1992) *J. Biol. Chem.* 267, 12927–12932
13. Stark, H., Rodinina, M., Rinkeappel, J., Brimaconbe, R., Wintemerry, W., and Vanhee, M. (1997) *Nature* 389, 403–406
14. Wilson, K. S., and Noller, H. F. (1996) *Cell* 92, 131–159
15. Schneider, G., Anke, H., and Steenoz, O. (1995) *Nat. Prod. Lett.* 7, 309–316
16. Cova, S. J., Puar, M. S., Phife, D. W., Terracciano, S. J., and Patel, M. (1995) *J. Antibiot. (Tokyo)* 48, 1171–1172
17. Kimmons, O. S., Chalk, P. A., Jackson, H. C., Middleton, R. F., Shuttleworth, A., Rudd, B. A. M., Jones, C. A., Noble, H. M., Wildman, H. G., Dave, M. J., Styli, C., Sidebothom, P. J., Latom, B., Lynn, S., and Hayes, M. V. (1998) *J. Antibiot. (Tokyo)* 51, 41–49
18. Justice, M. C., Hsu, M. J., Tse, E., Ku, T., Balkovec, J., Schmatz, D., and Nielsen, J. (1996) *J. Biol. Chem.* 271, 3148–3151
19. Santos, C., and Ballesta, J. P. G. (1994) *J. Biol. Chem.* 269, 15689–15696
20. Guthrie, C., and Fink, G. R. (eds) (1991) *Guide to Yeast Genetics and Molecular Biology*, Academic Press, Inc., San Diego, CA
21. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* 153, 163–168
22. Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 2760–2767
23. MacConnell, W. P., and Kaplan, N. O. (1982) *J. Biol. Chem.* 257, 5359–5366
24. Rothstein, R. (1991) *Methods Enzymol.* 253, 281–301
25. Carrasco, L., and Vazquez, D. (1973) *Biochim. Biophys. Acta* 319, 209–215
26. Maozed, D., Robertson, J. D., and Noller, H. F. (1988) *Nature* 334, 362–364
27. Fried, H. M., and Warner, J. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 238–242
28. Fried, H. M., and Warner, J. R. (1982) *Nucleic Acids Res.* 10, 3133–3148
29. Larkin, J. C., and Woolfard, J. L. J. (1983) *Nucleic Acids Res.* 11, 403–420
30. Kafer, N. F., Fried, H. M., Schwindinger, W. F., Jasim, M., and Warner, J. R. (1983) *Nucleic Acids Res.* 11, 3123–3135
31. Kawai, S., Murao, S., Mochizuki, M., Shibuya, I., Yano, K., and Takagi, M. (1992) *J. Bacterial.* 174, 254–262