

Role of the ribosomal stalk components in the resistance of *Aspergillus fumigatus* to the sordarin antifungals

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Summary

Aspergillus fumigatus, an important human nosocomial pathogen, is resistant to sordarin derivatives, a new family of antifungals that inhibit protein synthesis by interaction with the EF-2–ribosomal stalk complex. To explore the role of the *A. fumigatus* ribosome in the resistance mechanism, the fungal stalk proteins were biochemically and genetically characterized and expressed in the sensitive *Saccharomyces cerevisiae*. Two acidic phosphoproteins homologous to the 12 kDa P1 and P2 proteins described in other organisms were found together with the 34 kDa P0 protein, the third stalk component. The genes encoding each fungal stalk protein were expressed in mutant *S. cerevisiae* strains lacking the equivalent proteins. Both AfP1 and AfP2 proteins interact with their yeast counterparts of the opposite type and bind to the ribosomal particles in the presence of either the *S. cerevisiae* or the *A. fumigatus* P0 protein. The *A. fumigatus* acidic phosphoproteins did not alter the yeast ribosome sordarin sensitivity. On the contrary, the presence of the fungal P0 induces *in vivo* and *in vitro* resistance to sordarin derivatives when present in the yeast ribosome. The mutations A¹¹⁷→E, P¹²²→R and G¹²⁴→V in *A. fumigatus* P0 reduce the resistance capacity of the protein. An *S. cerevisiae* strain with the complete ribosomal stalk of *A. fumigatus* was obtained, which could be useful for the screening of new antifungals against this pathogenic fungus.

Introduction

A new family of antifungal compounds derived from sordarin has been described recently that inhibit the translo-

cation step of protein synthesis in yeasts and other filamentous fungi (Capa *et al.*, 1998; Justice *et al.*, 1998). The sordarin derivatives block the interaction of elongation factor EF-2 with the ribosome, depending on the ribosomal stalk for this inhibiting effect (Gómez-Lorenzo and García-Bustos, 1998; Justice *et al.*, 1999).

The stalk is a characteristic structure of the large ribosomal subunit involved in the interaction with the soluble factors during translation. The ribosomal stalk is universally composed of a pentameric complex of four 12 kDa acidic polypeptides and one larger RNA-binding protein (Uchiumi *et al.*, 1987; Ballesta and Remacha, 1996). In eukaryotes, there are two 12 kDa acidic protein families, P1 and P2, and the RNA-binding component, P0, has evolved to a larger protein by gaining an additional carboxyl domain not found in bacterial L10. All the eukaryotic stalk components, P0, P1 and P2, are phosphorylated in the ribosome (for a review, see Ballesta *et al.*, 1999). This modification has been demonstrated to be important for the degradation of the acidic proteins (Nusspaumer *et al.*, 2000), and it might have a functional role as well (Zambrano *et al.*, 1997; Vard *et al.*, 1997; Rodríguez-Gabriel *et al.*, 1998).

In some eukaryotes, there are several members in each acidic protein family (Ballesta and Remacha, 1996); thus, in *Saccharomyces cerevisiae*, two proteins of each type were described: P1 α –P1 β and P2 α –P2 β (Newton *et al.*, 1990). At least one protein from each family, probably present as P1/P2 heterodimers (Ballesta *et al.*, 2000), is necessary to form a stable stalk (Remacha *et al.*, 1992). The stalk has been shown to be particularly flexible, as confirmed by recent cryoelectron microscopy studies (Agrawal *et al.*, 1999; Stark *et al.*, 2000). In *S. cerevisiae* ribosomes, the stalk has been detected by this technique only when it forms a complex with EF-2 and sordarin, which seems to immobilize this structure (Gómez-Lorenzo *et al.*, 2000).

As commented previously, the EF-2 blocking effect of sordarin derivatives is conditioned by the ribosomal stalk. Thus, mutations in the P0 protein, the only essential component for stalk formation in yeast (Santos and Ballesta, 1994), can affect the sordarin inhibitory activity. Several P0 mutations have been mapped that induce resistance to the sordarin derivatives (Gómez-Lorenzo and García-Bustos, 1998; Justice *et al.*, 1999). Moreover, the level of

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P0-induced resistance to these antifungal agents has been shown to depend on the presence of the P1 α and P2 β proteins in the ribosome (Gómez-Lorenzo and García-Bustos, 1998).

The sordarin derivatives are very active inhibitors for all *Candida* species and other fungal pathogens, but several species of clinical interest have been described that are resistant to these compounds (Herreros *et al.*, 1998). The opportunistic human pathogen *Aspergillus fumigatus*, a filamentous fungus that causes serious nosocomial infections in immunosuppressed patients (Fridkin and Jarvis, 1996), is one of these sordarin-resistant organisms. The aim of this study is to analyse the role that the ribosomal stalk components might have in *A. fumigatus* resistance to sordarin antifungals.

For this purpose, *Aspergillus* stalk components were biochemically characterized, cloned and expressed in sordarin-sensitive *S. cerevisiae* strains lacking the equivalent proteins. These yeast strains were tested to check whether the *A. fumigatus* proteins were able to induce resistance to the sordarin antifungals.

Results

Characterization of the 12 kDa acidic ribosomal proteins of *Aspergillus fumigatus*

Analysis of *A. fumigatus* ribosomes purified by isoelectrofocusing (pH range 2–5) showed the presence of two main bands (Fig. 1). The band with a lower isoelectric point, moving to an intermediate position between *S. cerevisiae* P1 β and P2 α , was assumed to belong to the P1 type and called AfP1. The more basic band, moving above P2 β , was considered to be the P2 type AfP2 (Fig. 1). Treatment of the sample with alkaline phos-

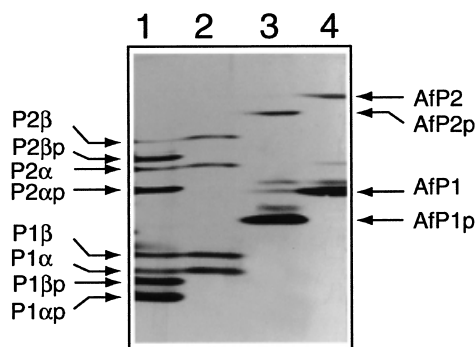


Fig. 1. Acidic protein composition of the *A. fumigatus* ribosomes. Ribosomes (50 μ g) from *S. cerevisiae* (lanes 1 and 2), used as reference, and *A. fumigatus* (lanes 3 and 4) were resolved by isoelectrofocusing either directly (lanes 1 and 3) or after alkaline phosphatase treatment (lanes 2 and 4). The resolved proteins were detected by silver staining. The position of the different proteins is indicated. The phosphorylated forms of the proteins are indicated by a 'p' after the protein name.

phatase increased the pI of both proteins, indicating that the AfP1 and AfP2 proteins are phosphorylated on the ribosomes (Fig. 1).

A further characterization of the proteins was carried out by amino acid sequencing (Table 1). Sequence comparison with proteins in databases showed that the AfP2 N-terminal obtained by Edman degradation is identical to the equivalent sequence of P2 protein from *Alternaria alternata* (Achatz *et al.*, 1995) and shows 80% sequence identity to the RLA3 and RLA4 proteins from *Cladosporium herbarum* (Achatz *et al.*, 1995; Zhang *et al.*, 1995), and the YP2 α and YP2 β proteins from yeast (Remacha *et al.*, 1988). In contrast, no N-terminal sequence could be obtained from AfP1 by this method, suggesting that, as has been described previously in P1 proteins from yeast (Santos *et al.*, 1993) and other eukaryotes (van Aghoven *et al.*, 1978), the equivalent *Aspergillus* protein carries an acetylated amino-terminal. Therefore, to identify the protein chemically, a sample of AfP1 resolved by isoelectrofocusing was tryptically digested, and some peptides were analysed by mass spectrometry. With this technique, the amino acid sequence of two peptides was obtained (Table 1). Peptide 1 from the AfP1 protein showed 75% identity to the P1 of *A. alternata* (amino acids 15–26), and peptide 2 was 78% identical to the sequence from amino acids 36–49 of the same protein of *A. alternata* and *C. herbarum* (Achatz *et al.*, 1995).

Characteristics of the *A. fumigatus* P1/P2 protein-encoding genes

Comparison of the *AfRPP1* gene and the corresponding cDNA nucleotide sequences, cloned as described in *Experimental procedures*, showed the presence of three introns of 162, 105 and 61 bp at nucleotide (nt) positions 70, 310 and 557 respectively (Fig. 2). The three introns bear the typical 5' and 3' sequences required for splicing, as well as the internal sequence involved in lariat formation. No TATA or CAAT boxes were detected in the *AfRPP1* 5' upstream region, but a CT-rich region was present 35 nt upstream from the ATG start codon, which could indicate the transcription starting point of the gene. A putative polyadenylation sequence, ATAATA, was present 100 nt upstream from the poly(A).

Two introns were found in the *AfRPP2* gene (Fig. 2). One of them is located from nt –89 to nt –8 in the 5' untranslated region of the gene and the other from nt 160 to nt 230 (Fig. 2). Both introns possess the 5' and 3' consensus sequences for splicing of fungal introns (Gurr *et al.*, 1987) as well as the internal sequence (NNCT-PuAPy) required for lariat formation (Unkless, 1992). In the 5' upstream region, two CAAT sequences were located at positions –216 and –729, although no TATA box was present. In the 3' downstream region of the gene, a

Table 1. Amino acid sequence of peptides derived from *A. fumigatus* P1 and P2 proteins.

Protein	Experimental amino acid sequence	Deduced amino acid sequence from the gene
AfP2 ^a	MKHLAAYLLL	MKHLAAYLLL
AfP1 ^b	Peptide 1: (I/L)-A-D-D-(R/GV)-E-(I/L)-T-A-D-K Peptide 2: D-V-E-P-(I/L)-(EG/AD/SV/W)-T-S-(I/L)-(F/Mox)-A-K	LADDGVEITADK DVEPIWTSLSFAK

a. Amino acid sequences of the N-terminal end. The sequence was determined by Edman degradation.

b. Internal peptides. Sequences determined by mass spectrometry.

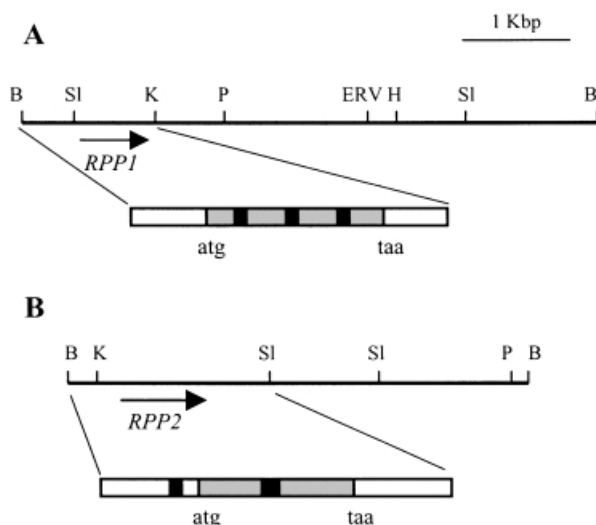


Fig. 2. Schematic representation of the cloned genomic DNA fragments containing the *AfRPP1* (A) and *AfRPP2* (B) genes from *A. fumigatus*. The arrows indicate the position of the open reading frames. The introns are in black and the exons in grey. The endonuclease restriction sites marked are: B, *Bam*HI; SI, *Sal*I, H, *Hind*III; ERV, *Eco*RV; P, *Pst*I; K, *Kpn*I.

putative polyadenylation sequence ATATAA was located at position +559, 54 nt upstream from the polyadenylation starting point.

Characteristics of the *A. fumigatus* P1/P2 proteins

The amino acid sequence deduced from the cloned cDNAs showed similar molecular weights for AfP1 and AfP2 (11 128.4 and 11 141.34 Da respectively) and a theoretical isoelectric point of 4.27 for AfP1 and 4.43 for AfP2. Both proteins contain 111 amino acid showing 51% sequence identity. Comparison of the amino acid sequences of P1 and P2 from *A. fumigatus* and *S. cerevisiae* (Fig. 3) confirmed the higher similarity of proteins of the same type that have an overall average identity of 60%, which decreases to 42% in proteins of a different type. The fungal proteins also contain the same carboxyl end (last 12 amino acids), which makes them cross-react with the monoclonal antibody 3BH5 (Vilella *et al.*, 1991), specific for this highly conserved peptide (data not shown).

Expression of the AfP1 and AfP2 proteins in *S. cerevisiae*

To test the effect of the *A. fumigatus* acidic proteins on *S. cerevisiae* ribosomes, yeast strains lacking different stalk proteins were transformed with plasmids carrying the fungal proteins under the control of the 5' upstream and 3' downstream regions of the corresponding *S. cerevisiae* genes. A summary of the strains and plasmids used is shown in Table 2.

In a first series of tests (Table 2A), yeast strains lacking either P1 α and P1 β proteins (strain D67) or P2 α and P2 β proteins (strain D45) were used. In both strains, the acidic proteins present in the cell are unable to bind to the ribosome, which are totally deprived of these components (Remacha *et al.*, 1992). When transformed with the corresponding plasmids, the expressed *A. fumigatus* proteins were found stably bound to the yeast ribosome, at the same time stimulating the binding of the yeast proteins (Fig. 4). Thus, the binding of AfP2 to D45 ribosomes allowed the interaction of both *S. cerevisiae* P1 α and P1 β proteins with the ribosome, whereas AfP1 tended to stimulate the binding of P2 β . Moreover, the expression of these *A. fumigatus* proteins in the double disruptant yeast strains D45 and D67 induced a stimulation of the growth rate (Table 2), indicating the positive effect of these heterologous proteins on the *S. cerevisiae* ribosomes.

A second set of tests was performed using *S. cerevisiae* strains in which the P0 protein gene was under the control of the GAL1 promoter (Table 2B). As P0 is an essential protein, these cells cannot grow in glucose media unless an alternative P0 gene is provided in a transforming plasmid (Santos and Ballesta, 1994). These strains were transformed with plasmids carrying different *A. fumigatus* stalk component genes, including the fungal P0, and grown in glucose medium. As shown previously, the fungal P0 protein can replace the yeast protein in wild-type strains, although it causes a reduction in the growth rate and some alteration in stalk composition (Rodríguez-Gabriel *et al.*, 2000). The results obtained with the stalk-defective mutants D45dGP0 and D67dGP0 are similar on the whole to those shown earlier with the equivalent D45 and D67 strains. As shown in Fig. 4B, in the ribosomes with the AfP0 and AfP1 proteins, the *S. cerevisiae* P2 β was stably bound to the particles, but the P2 α protein was

	10	20	30	40	50	60	70	80	90	100
P1 α	MST-ESALSTAALILADSEIEISSEKLLTLTNAANV-PVENIWADIFAKALDQNLKDLLVNF-SAGAAAPAGVAGGVAGGEAGEAE---AEKEEBEAK-EESDDDMGFLFD									
AfP1	MSTAELACSYAALILADDGVEITADKIQTLLGAAKVADVEPIWTSLFAKALEGKDIKDLLTNVSGGAAAPAAV-GGAAAGAAAPAEAAAEKKEEEK-EESDEDMGFLFD									
P1 β	MSDSII--SFAAFILADAGLEITSDNLLTITKAAG-ANVDNVADVYAKALEGKDLKEILSGFHNAG---PVAG-AGAASGAAAGGDAAEKEEKEEAEESDDDMGFLFD									
	10	20	30	40	50	60	70	80	90	100
P2 α	MKYLAAYLLLNAAGNT-PDATKIKAIKESVGIIEDEKVSLSALEGK-SVDELITGNEKLAAPV----AAGPASAGGAAASGDAAAEKEEKEEAEESDDDMGFLFD									
AfP2	MKHLAAYLLLALAGNTSPSSSEVDKAVLSSVGIDADEERLNKLAIELEGK-DLQELIAEGSTKLASVPSGGAAAAAPAAAGAAAGGAAAPAEKEEKEE-EESDEDMGFLFD									
P2 β	MKYLAAYLLLQGGNAAPSADIKAVVESVGAEDVDEARINELLSSLEGKSLLEETIAEGQKFKATVPTGGASSAAAGAAGAAAGGDA--AEKEEKEE-EESDDDMGFLFD									
	10	20	30	40	50	60	70	80	90	100

Fig. 3. Comparison of the amino acid sequence of the AfP1 and AfP2 proteins from *A. fumigatus* with the yeast proteins of the same group. Identical amino acids are indicated by (:) and homologous ones by (.). Comparison was carried out using the ALIGN program.

Table 2. Doubling time of the *S. cerevisiae* strains expressing the *A. fumigatus* P proteins grown in glucose liquid rich medium.

<i>S. cerevisiae</i> strain	Missing yeast stalk proteins	Transforming plasmid	Protein composition of the stalk	Doubling time (min)
A				
W303	None		P0, P1 α , P1 β , P2 α , P2 β	90
D45	P2 α , P2 β		P0	200
D45-AfP2	P2 α , P2 β	pFLTAfP2	P0, P1 α , P1 β , AfP2	120
D67	P1 α , P1 β		P0	180
D67-AfP1	P1 α , P1 β	pFLHAfP1	P0, P2 β , AfP1	120
B				
W303dGP0-AfP0	None	pFLHAfP0	AfP0, P1 α , P1 β ^a , P2 α ^a , P2 β	150
D45dGP0-AfP0P2	P2 α , P2 β	pFLTAfP0P2	AfP0, AfP2, P1 α , P1 β	180
D67dGP0AfP0P1	P1 α , P1 β	pFLHAfP0P1	AfP0, AfP1, P2 β	180
D4567dGP0AfP0P1P2	P1 α , P1 β , P2 α , P2 β	pFLTAfP0P1P2	AfP0, AfP1, AfP2	170

a. The amount of these proteins in the ribosomes is lower compared with the other two proteins.

not. In contrast, in *S. cerevisiae* strains expressing AfP0 and AfP2 proteins, both yeast P1 proteins P1 α and P1 β were present in the ribosomes. The fungal AfP2 seems to be able to replace both P2 proteins, inducing the interaction of yeast P1 α and P1 β , whereas AfP1 only stimulates the binding of P2 β , regardless of the P0 protein present in the ribosome.

To test whether the complete *A. fumigatus* ribosomal stalk was functional in *S. cerevisiae*, an *S. cerevisiae* D4567dGP0 lacking the four acidic protein genes and carrying the *RPP0* gene under the *GAL1* promoter was transformed with the plasmid pFLT-AfP0P1P2. Several D4567dGP0-AfP0P1P2 transformants were selected in glucose-rich medium plates. Ribosomes purified from these transformants were subjected to isoelectrofocusing (Fig. 4C) showing a normal *A. fumigatus* ribosomal stalk composed of the AfP0, AfP1 and AfP2 proteins.

In general, all strains carrying the fungal P0 grow more slowly than the equivalent strains containing the yeast P0 protein in spite of the presence of some yeast acidic proteins (Table 2).

Effect of A. fumigatus stalk proteins in the resistance of S. cerevisiae strains to sordarin derivatives

The sordarin inhibition curves carried out on the yeast strains that express different fungal proteins (Table 2) indicate that the expression of only AfP1 (D67-AfP1) or AfP2 (D45-AfP2) in *S. cerevisiae* has no effect on the resistance level (Fig. 5). In contrast, the expression of the AfP0 protein alone, in the presence of all the acidic yeast proteins (W303dGP0-AfP0), resulted in a 10-fold increase in the IC₅₀ value for sordarin compared with the wild-type strain W303. The additional expression of either AfP1 or AfP2 together with the AfP0, when complementing yeast proteins are also present (D45dGP0-P0P1Af and D67dGP0-AfP0P2), does not have any supplementary effect on the resistance. A similar resistance level is displayed when only the three *A. fumigatus* proteins are expressed in strain D4567dGP0, forming a complete fungal ribosomal stalk in the yeast ribosome.

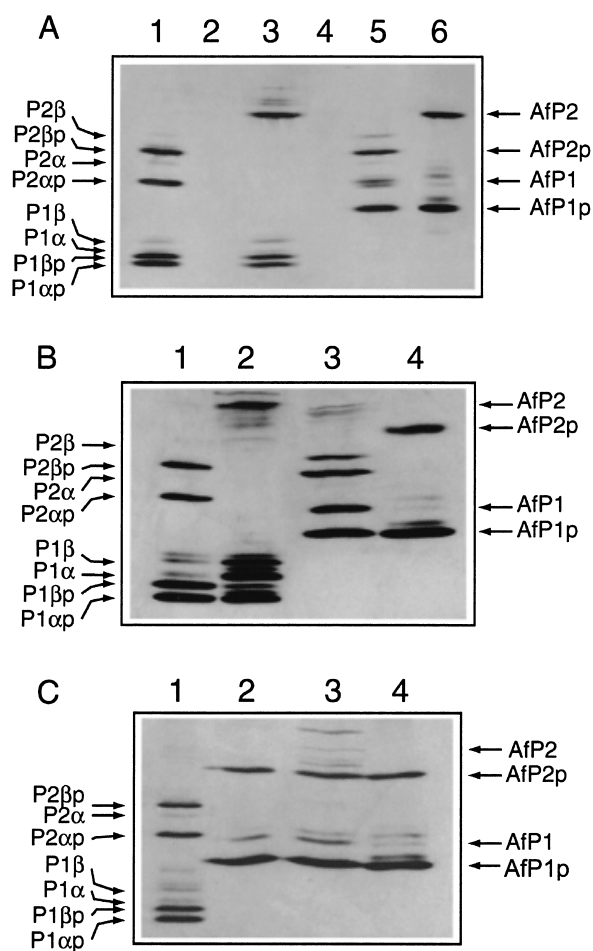


Fig. 4. Analysis of the acidic protein content of ribosomes from yeast strains expressing the *A. fumigatus* stalk components. Purified ribosomes (50 µg) were resolved by isoelectrofocusing (pH range 2–5). A. Ribosomes from yeast strains W303 (1), D45 (2), D45-AfP2 (3), D67 (4), D67-AfP1 (5) and *A. fumigatus* (6). B. Ribosomes from yeast strains W303 (1), D45dGP0-AfP0P2 (2), D67dGP0-AfP0P1 (3) and *A. fumigatus* (4). C. Ribosomes from strains W303 (1), D4567dGP0-AfP0P1P2 (2 and 3) and *A. fumigatus* (4). Proteins are marked as in Fig. 1.

Analysis of the AfP0 amino acids involved in resistance to sordarin

A number of mutations have been mapped in a region around position 140 in *S. cerevisiae* P0 that induce resistance to sordarin derivatives (Gómez-Lorenzo and García-Bustos, 1998; Justice *et al.*, 1999). A comparison of the *A. fumigatus* and *S. cerevisiae* P0 amino acid sequences indicated that the region from position 130–147 is identical in both proteins (Fig. 6), but three positions, amino acids 117, 122 and 124, not far from the previously defined resistance region, were different in both sequences. To test whether these amino acids have

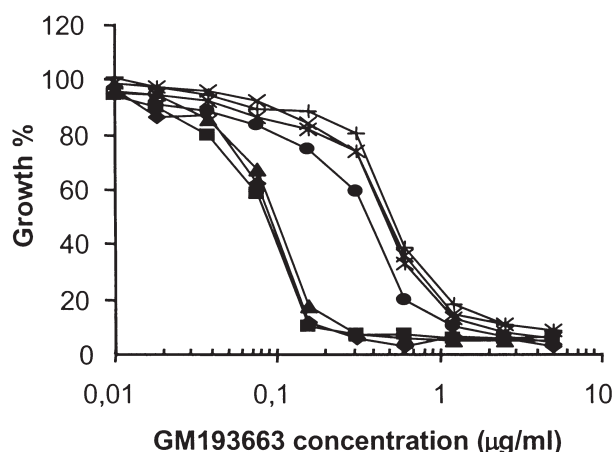


Fig. 5. Effect of *A. fumigatus* stalk components on resistance to the sordarin derivative GM193663 in *S. cerevisiae*. Strains D67AfP1 (closed squares), D45AfP2 (closed triangles), D67dGP0-AfP0P1 (x), D45dGP0-AfP0P2 (*), D4567dGP0-AfP0P1P2 (closed circles) and W303dGP0-AfP0 (+) were grown in glucose liquid medium in the presence of increasing concentrations of the inhibitor. *S. cerevisiae* W303 (closed diamond) was used as a sensitive control.

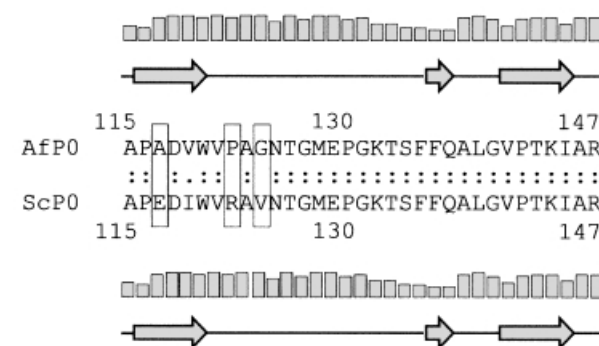


Fig. 6. Amino acid sequence and secondary structure of the *A. fumigatus* and *S. cerevisiae* P0 proteins (AfP0 and ScP0) in the region involved in sordarin derivative resistance. The amino acids that are different and afterwards mutated in the fungal protein have been boxed. (↔) alpha strand, (—) coil. Bars indicate the confidence of secondary structure prediction.

a role in the resistance of *A. fumigatus*, they were mutated to those present in the *S. cerevisiae* sequence, namely, A¹¹⁷→E, P¹²²→R and G¹²⁴→V. The mutated P0 gene was cloned in the pFL plasmid yielding the recombinant plasmid pFLH-AfP0*, which was used to transform *S. cerevisiae* W303dGP0. The transformant W303dGP0-AfP0* strain grew in glucose medium with a doubling time similar to that of W303dGP0-AfP0, which expresses the native *A. fumigatus* P0, indicating that the *Aspergillus* mutant protein AfP0* was functional. When tested for sordarin resistance, W303dGP0-AfP0* showed an IC₅₀ of 0.45 µM, higher than the IC₅₀ of the wild -type W303

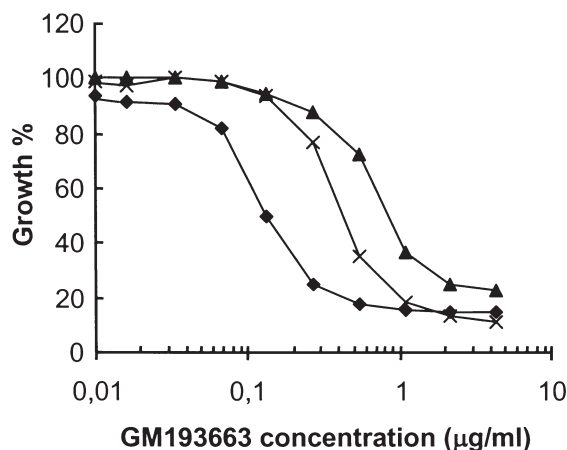


Fig. 7. Effect of *S. cerevisiae* P0 mutations on the resistance to sordarin derivatives. *S. cerevisiae* W303dGP0-P0Af* (x), expressing a P0 protein carrying the mutations A¹¹⁷→E, P¹²²→R and G¹²⁴→V, was grown in the presence of increasing concentrations of GM193663. W303dGP0-P0Af (closed triangles), expressing the wild-type fungal P0 and *S. cerevisiae* W303 (closed diamonds) expressing the native yeast P0 protein were used as controls.

strain (0.1 µM) but lower than that of W303dGP0-AfP0 (0.8 µM) (Fig. 7).

Discussion

Aspergillus fumigatus, one of the organisms most frequently responsible for nosocomial infections, is resistant to the new antifungal sordarin derivatives (Herreros *et al.*, 1998). The *in vitro* protein synthesis tests clearly indicated that the resistance of *A. fumigatus* is associated with the protein synthesis machinery and not with either transport or inhibitor inactivation mechanisms. As the sordarin derivative target is the EF-2-ribosomal stalk complex (Gómez-Lorenzo and García-Bustos, 1998; Justice *et al.*, 1999), we focused our attention on the role that the stalk components might have in *A. fumigatus* resistance.

In contrast to yeasts, only two acidic proteins have been detected in the *A. fumigatus* ribosome, AfP1 and AfP2, one from each standard eukaryotic stalk P protein family. Both proteins are phosphorylated in the ribosome as in most eukaryotic organisms (Ballesta and Remacha, 1996). The AfP1 and AfP2 proteins have very high homology with their counterparts from the filamentous fungi *C. herbarum* and *A. alternata*, which have been identified as allergens (Achatz *et al.*, 1995). In fact, the highly immunogenic properties of the eukaryotic stalk proteins are well known, as they were also found as antigens for sera from patients with systemic lupus erythematosus (Francoeur *et al.*, 1985), Chagas' disease (Levin *et al.*, 1989) and leishmaniasis (Soto *et al.*, 1995).

The cDNAs and the genes encoding the AfP1 and AfP2 proteins were cloned and sequenced. The *AfRPP1* gene possesses three small introns in the coding region, whereas the *AfRPP2* gene carries two small introns, one of them in the 5' untranslated region of the gene. The presence of small introns in the genes of filamentous fungi is usual, but the presence of one intron in the 5' non-coding region is less common.

The strategy followed to establish the role of the fungal stalk in sordarin resistance was to express the *A. fumigatus* stalk components in *S. cerevisiae* strains and test the effect on their sensitivity to the inhibitors. Several *S. cerevisiae* strains were used, some of them lacking one or more of their own stalk acidic proteins. In this way, the ability of the fungal proteins to replace the endogenous ones was also tested. The available data indicate that the eukaryotic acidic proteins are assembled into the ribosome as a P1/P2 heterodimer, which interacts with P0 primarily through the P1 protein (Ballesta *et al.*, 2000; Guarinos *et al.*, 2001). The results in this report show that AfP1 and AfP2 bind to the ribosome in a stable way, suggesting that they were able to form heterodimers with the yeast acidic proteins. AfP2 seems to be able to interact with both P1α and P1β proteins, forming AfP2/P1α and AfP2/P1β heterodimers, which bind to P0, resulting in ribosomes carrying the three acidic proteins. In contrast, AfP1 was preferentially bound to the yeast P2β protein and, to a lesser extent, to P2α. The presence of the fungal protein stimulates ribosome activity in *S. cerevisiae* D45 and D67 by inducing the binding of the yeast proteins and the formation of a complete stalk (Table 2). These results suggest a closer similarity of AfP1 to yeast P1α, which is the partner of P2β in the yeast ribosome. However, this functional proximity is not obvious from the comparison with both yeast P1 proteins, which show primary and secondary structures similar to those of AfP1.

Aspergillus fumigatus P0 can also be incorporated into the yeast ribosome, slowing down the growth rate and altering the stalk composition, as yeast proteins P1β and P2α bind poorly to the fungal protein. In the case of the defective D45dGP0 and D67dGP0 strains, the negative effect of both AfP0 and one of the fungal acidic proteins is compensated by the presence of the partner yeast proteins, resulting in no effect on growth (Table 2). Using the D4567dGP0 strain, it was possible to construct an *S. cerevisiae* ribosome carrying a complete *A. fumigatus* stalk, which seems to be reasonably active, as the cells can grow with a doubling time of around 170 min. This slow growth suggests that the interaction of the yeast elongation factor EF-2 with the fungal stalk is less efficient than it is with the homologous one.

The results from the sordarin resistance tests using strains expressing different *A. fumigatus* proteins indicate that only P0 affects the resistance to sordarin. The

expression of only one acidic fungal protein, either AfP1 or AfP2 in yeast, had no effect on the level of resistance of *S. cerevisiae* to the antibiotic, nor did it increase the resistance level induced by AfP0. It seems therefore that only P0 is directly involved in the EF-2–stalk–sordarin complex. These data, together with those obtained previously from the *S. cerevisiae* resistant mutants (Gómez-Lorenzo and García-Bustos, 1998; Justice *et al.*, 1999), suggest that P0 plays a more important role in stalk function than the acidic proteins. This concept is in agreement with the non-essential character of these last components, which are proposed to play a modulating effect in ribosomal function (Ballesta *et al.*, 2000).

The identification of P0 mutations inducing *S. cerevisiae* resistance to sordarin derivatives defined a region about positions 135–145 (Gómez-Lorenzo and García-Bustos, 1998; Justice *et al.*, 1999) where the antibiotic and/or the EF-2 interact. Our results indicate that this region is larger than initially proposed. Replacing the amino acids around position 120 in the fungal proteins with those from *S. cerevisiae*, namely A¹¹⁷→E, P¹²²→R and G¹²⁴→V, resulted in a clear reduction in the resistance induced by the wild-type AfP0. These results therefore indicate that the part of P0 comprising around positions 115–140 must somehow be related to the binding of the sordarin derivative inhibitors and probably of EF-2. In fact, a similar domain from human P0 has also been shown to be responsible for inducing sordarin resistance to yeast (M. A. Rodríguez Gabriel, M. Remacha and J. P. G. Ballesta, unpublished results). However, the sensitivity induced by these mutations is even lower than that of the wild-type *S. cerevisiae* P0, which indicates that some other structural features participate in building up the resistance caused by wild-type AfP0.

The highly conserved C-terminal of the stalk proteins has been assumed to be the domain directly involved in the interaction with the supernatant factors. Our results are consistent with the hypothesis that the domain from position 115–140 in protein P0 must also be related to binding of the EF-2 during translocation. Moreover, this domain must be responsible for the different protein conformation that prevents translocation inhibition mediated by sordarin antifungals in the yeast strains carrying the AfP0 protein and, in general, in all resistant organisms.

Nevertheless, it must be indicated that, as in *S. cerevisiae* resistant mutants, the elongation factor EF-2 also seems to participate in the resistance of *A. fumigatus* to sordarin derivatives. Unfortunately, our attempts to express the fungal factor in yeast have so far shown very low complementing activity. The yeast cells expressing the fungal factor grow very slowly, which makes it difficult to perform accurate estimations of their sordarin resistance properties.

Experimental procedures

Strains and growth conditions

Aspergillus fumigatus 48238E strain was obtained from Glaxo Wellcome Spain. *S. cerevisiae* W303 strains have been described previously (Remacha *et al.*, 1992; 1995; Santos and Ballesta, 1994). *Escherichia coli* XL1-Blue MRF' and SOLR strains from Stratagene were used for the propagation of a cDNA library and for plasmid excision respectively. *E. coli* DH5 α was used for plasmid maintenance and preparation. Bacterial strains were grown on LB medium.

Yeasts were grown in either YEP (1% yeast extract, 2% peptone) or YNB (0.67% yeast nitrogen base without amino acids, 40 $\mu\text{g mL}^{-1}$ required amino acids) medium supplemented with either 2% glucose or 2% galactose. When conditional null mutants were shifted from glucose to galactose medium, cells were allowed to grow for at least 10 generations. *A. fumigatus* were grown in YEPD medium at 30°C with orbital shaking.

Enzymes and reagents

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, the DNA polymerase I Klenow fragment and calf intestinal alkaline phosphatase were purchased from MBI Fermentas, Amersham Pharmacia Biotech and Roche Molecular Biochemicals. [α -³²P]-dCTP (3000 Ci mmol⁻¹) and [γ -³²P]-dATP (3000 Ci mmol⁻¹) were obtained from Amersham Pharmacia Biotech.

DNA isolation and analysis

Aspergillus fumigatus genomic DNA was isolated as described by Tang *et al.* (1992). DNA manipulations, Southern analysis and hybridization with radiolabelled probes were performed as described by Sambrook *et al.* (1989).

Preparation of radioactive probes

DNA probes were labelled by random priming with 25 μCi of [α -³²P]-dCTP to a specific activity of 10⁷–10⁸ c.p.m. μg^{-1} , using the High Prime system (Roche Molecular Biochemicals). Oligonucleotide probes were labelled at the 5' end with 25 μCi of [γ -³²P]-dATP using the T4 polynucleotide kinase.

Genomic and cDNA library construction and screening

The *A. fumigatus* cDNA library in λ -ZAP was kindly provided by Dr García-Bustos (Glaxo Wellcome Research Department, Madrid, Spain). Genomic DNA libraries were constructed from *Bam*HI-digested *A. fumigatus* DNA resolved in agarose gels. DNA fragments (3–5 kb), purified using the Gene clean system (BIO-101), were cloned in pUC19. DH5 α *E. coli* cells were transformed with the plasmids, and screening was carried out using the radioactively labelled probes by colony hybridization (Sambrook *et al.*, 1989).

DNA mapping and sequencing

The plasmids from positive clones were mapped by Southern blotting of plasmids digested with several restriction endonucleases using the probes used in the screening. Sequencing of the cloned genes was carried out by automatic sequencing (ABI 377).

Sequence analysis

Nucleotide and protein sequence comparisons were carried out using the Advanced BLAST search algorithm (Altschul *et al.*, 1997). DNA and protein sequence alignment and comparisons were performed using the CLUSTAL W (version 1.7) (Thompson *et al.*, 1994) and LALIGN programs.

Cloning of the RPP2 and RPP1 genes of *A. fumigatus*

The oligonucleotide AfP2-2 (5'-atgaagcacctSgcYgc-3') derived from the N-terminal amino acid sequence of AfP2 was used to screen an *A. fumigatus* cDNA library. Positive clones were subcloned into Bluescript and sequenced. Two of them encoded proteins homologous to the ribosomal P2 from other organisms.

The *AfRPP2* gene encoding protein AfP2 was cloned by screening a subgenomic DNA library using the *AfP2* cDNA as a probe. Two positive clones, pUCAfP2, were obtained that carried a 1.9 kb *Bam*HI–*Sal*I DNA fragment containing the *RPP2* gene, which was subcloned into pUC19 and sequenced.

A similar strategy to the one described for *AfRPP2* failed in the case of the cDNA encoding AfP1. The cloning of *AfRPP1* was attempted directly from the genomic DNA. For this purpose, a Southern blot analysis of *A. fumigatus* DNA was carried out using three different probes: (i) *AfRPP2* cDNA; (ii) *AfRPP0* cDNA; and (iii) a 300 bp *Xho*I–*Kpn*I DNA fragment from the *AfRPP2* cDNA, containing the most conserved sequence (120 nt) of both previous probes, *AfRPP2* and the *AfPPO* cDNAs, which correspond to the conserved carboxyl end of the proteins. The hybridizations carried out with the two first probes were performed under high-stringency conditions (Sambrook *et al.*, 1989) and yielded single bands, which clearly defined the position of the genomic DNA fragments containing the *AfRPP2* and *AfRPP0* genes respectively. In contrast, hybridization with the third probe, carried out under low-stringency conditions (washing the membranes twice with 0.1% SDS, 2 × SSC; Sambrook *et al.*, 1989), revealed three different bands. Two of them corresponded to the previously identified *AfRPP2* and *AfRPP0* genes. The third band was used to prepare a library in the pUC19 plasmid, which was screened with the 300 bp *Xho*I–*Kpn*I DNA probe. A unique positive clone containing a 3.4 kb insert, pUCAfP1, was obtained. Restriction and Southern analysis of the insert located the *AfRPP1* gene in a 0.7 kb *Kpn*I–*Sal*I DNA fragment, which was confirmed by sequencing.

The cloned *AfRPP1* gene was used as a probe in the screening of the *A. fumigatus* cDNA library, and two clones encoding the *A. fumigatus* P1 protein were isolated.

The sequence data have been submitted to the GenBank database under accession numbers AF268869 for the *AfP1* gene and AF268870 for the *AfP2* gene.

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pFLT-AfP2. A 1 kb *Pst*I–*Pac*I DNA fragment containing the 5' upstream sequence and the coding region of the *RPP2B* *S. cerevisiae* gene was deleted from the pFL38RPP2 β plasmid (Nussbaumer *et al.*, 2000). This segment was replaced by an equivalent fragment containing the *A. fumigatus* *RPP2* gene coding region fused to the 5' upstream sequence of the yeast *RPP2 β* gene containing the appropriate *Pst*I and *Pac*I sites at the corresponding ends, which had been constructed by polymerase chain reaction (PCR). From the resulting 5.7 kb pFLU-AfP2 construct, a 1 kb *Pst*I–*Hind*III DNA fragment containing the *A. fumigatus* *RPP2* coding region under the 5' and 3' regulatory regions of the yeast *RPP2B* gene was cloned in the pFL39 plasmid (Bonneaud *et al.*, 1991) digested with the same restriction enzymes yielding the 5.3 kb pFLT-AfP2 plasmid.

pFLH-AfP1. This plasmid was constructed by cloning the *A. fumigatus* *RPP1* coding region under the 5' and 3' regulatory regions of the *S. cerevisiae* *RPP1A* gene between the *Eco*RI and *Bam*HI sites of pFL37 (Rodriguez-Gabriel *et al.*, 2000). The 5' upstream region of the yeast *RPP1A* gene was obtained by *Eco*RI–*Acc*I digestion of the BsP1 α plasmid (Zambrano *et al.*, 1997). The fungal *RPP1* coding region was generated by PCR with the oligonucleotides AfP1atg (5'-accatgtctactgccgagc-3') and AfP1taa (5'-ttagtcgaacagaccgaagc-3') using the pUC19AfP1 plasmid as a template. The 3' downstream region was amplified by PCR from BsP1 α with the oligonucleotides 1 α -5'T (5'-aagtcgccgactgttagaag-3') and Bs-down (5'-tgaattgtaatacactactat-3') and digested with *Bam*HI. The digested pFL37 plasmid, the yeast 5' and 3' flanking regions and the *Aspergillus* *AfP1* coding sequence were ligated simultaneously, resulting in the 5.4 kb pFLH-AfP1 plasmid.

pFLT-AfP0P2. A 2.8 kb *Eco*RI–*Xho*I DNA fragment from the BsAfP0 plasmid (Rodriguez-Gabriel *et al.*, 2000), containing the *AfRPP0* gene flanked by the 5' and 3' regulatory regions of the *S. cerevisiae* *P0* gene, was inserted into the equivalent sites of pFLT-AfP2, yielding the 8.1 kb pFLT-P0P2Af construct containing the fungal *RPP2* and *RPP0* genes controlled by the regulatory regions of the equivalent yeast genes.

pFLH-AfP0P1. A 2 kb *Eco*RI–*Bam*HI DNA fragment containing the *AfRPP1* gene under the regulatory regions of the yeast *RPP1A* gene was obtained from pFLH-AfP1 and cloned into BsAfP0 (Rodriguez-Gabriel *et al.*, 2000) digested with the same restriction enzymes. The resulting 7.6 kb BsAfP0P1 was digested with *Bam*HI and *Xho*I, yielding a 4.6 kb DNA fragment with both *AfRPP0* and *AfRPP1* genes, which was cloned into the *Bam*HI–*Sal*I pFL37 sites producing the 9.8 kb pFLH-AfP0P1 plasmid.

pFLT-AfP0P1P2. A 4.6 kb *Bam*HI–*Pst*I DNA fragment containing the *AfRPP0* and *AfRPP1* genes under the control of the equivalent *S. cerevisiae* flanking regions was inserted into the *Bam*HI–*Pst*I sites of pFLT-AfP2. The resulting 9.9 kb pFLT-AfP0P1P2 plasmid carries the three *Aspergillus* ribosomal stalk protein genes.

Mutation of *A. fumigatus* P0 at positions ($A^{117} \rightarrow E$, $P^{122} \rightarrow R$ and $G^{124} \rightarrow V$)

Two complementary oligonucleotides containing the desired mutations, AfP0-1 (5'-gggtgtgacagctctaaccagacgtcttcgggagcaatggcaccagc-3') and AfP0-2 (5'-gctcccgaagacgtctgggtagagctgtcaacaccggatgaaccc-3'), were designed to hybridize in the AfP0 cDNA region coding from amino acids 111–130. Using AfP0-1 and AfP0-2 as well as the appropriate oligonucleotides that hybridize in the 5' and 3' ends of the AfP0 cDNA, two DNA fragments were obtained by PCR, which were afterwards used in an overlapping PCR to obtain the complete *AfRPP0* coding region with the desired mutated nucleotides (AfP0*). The 2.5 kb PCR product, digested with the restriction endonucleases *Not*I and *Eco*RI, was cloned in pFLH digested with the same enzymes. The resulting plasmid, pFLH-AfP0*, was sequenced.

Cell transformation

Yeast strains were transformed by the lithium acetate method (Hill *et al.*, 1991). Bacterial transformations were carried out as described by Hanahan (1985).

Ribosome preparation and analysis

Aspergillus fumigatus mycelium was harvested by filtration, washed with sterilized distilled water and frozen into dry ice. The frozen mycelium was broken with a glass stick until it was reduced to a fine powder. To obtain the lysate, 10 ml of buffer 1 (100 mM Tris-HCl, pH 7.4, 20 mM KCl, 12.5 mM MgCl₂, 5 mM β -mercaptoethanol) with protease [1 mM phenylmethylsulphonyl fluoride (PMSF), 2.5 μ g ml⁻¹ leupeptin, pepstatin, antipain, aprotinin and chymostatin] and phosphatase (10 mM NaF) inhibitors were added mg⁻¹ mycelium. To purify the ribosomes from this lysate, the previously described protocol for the isolation of yeast ribosomes was followed (Rodríguez-Gabriel *et al.*, 2000). The ribosome yield was 9 mg g⁻¹ mycelium.

Protein analysis

Aspergillus fumigatus and yeast ribosomes were analysed by either 15% SDS-PAGE or isoelectrofocusing. Isoelectrofocusing was carried out as described previously (Zambrano *et al.*, 1997). For Western blotting, proteins in gels were transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes as described previously (Rodríguez-Gabriel *et al.*, 2000), and detected with specific antibodies (Vilella *et al.*, 1991).

Protein amino acid sequencing was carried out at our Protein Chemistry Service by either Edman degradation using an Applied Biosystem 447 automatic sequencer or mass spectrometry using a MALDI-TOF (Kompact Probe Instruments, Kratos-Shimadzu) and an ion-trap mass spectrometer LCQ (Finnigan; TermoQuest) as described previously (Pineiro *et al.*, 2001).

In vivo sordarin resistance assay

Sordarin resistance was tested in 96-well plates with 10⁵ cells per well in 100 μ l of rich medium supplemented with the derivative GM193663. Ten serial dilutions of the antibiotic were carried out starting with a concentration of 5 μ g ml⁻¹ in the first column. After 24 and 48 h of incubation at 30°C, the A₆₀₀ was measured and the IC₅₀ was calculated.

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