P1 and P2 protein heterodimer binding to the P0 protein of *Saccharomyces cerevisiae* is relatively non-specific and a source of ribosomal heterogeneity

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ABSTRACT

The ribosomal stalk is formed by four acidic phosphoproteins in *Saccharomyces cerevisiae*, P1α, P1β, P2α and P2β, which form two heterodimers, P1α/P2β and P1β/P2α, that preferentially bind to sites A and B of the P0 protein, respectively. Using mutant strains carrying only one of the four possible P1/P2 combinations, we found a specific phenotype associated to each P1/P2 pair, indicating that not all acidic P proteins play the same role. The absence of one P1/P2 heterodimer reduced the rate of cell growth by varying degrees, depending on the proteins missing. Synthesis of the 60S ribosomal subunit also decreased, particularly in strains carrying the unusual P1α–P2α or P1β–P2β heterodimers, although the distinct P1/P2 dimers are bound with similar affinity to the mutant ribosome. While in wild-type strains the B site bound P1β/P2α in a highly specific manner and the A site bound the four P proteins similarly, both the A and B binding sites efficiently bound practically any P1/P2 pair in mutant strains expressing truncated P0 proteins. The reported results support that while most ribosomes contain a P1α/P2β–P0–P1β/P2α structure in normal conditions, the stalk assembly mechanism can generate alternative compositions, which have been previously detected in the cell.

INTRODUCTION

The stalk is a functional domain of the large ribosomal subunit that is directly involved in the interaction and GTPase activity of several soluble factors during translation (1). In eukaryotes, the stalk is formed by a central 32-kDa protein, P0, which interacts through its N-terminal domain (NTD) with the highly conserved GTPase-associated region (GAR) of the large rRNA subunit, and with two heterodimers of the acidic 12-kDa P1 and P2 proteins, ultimately forming a P0–(P1/P2)$_2$ pentamer. Some lower eukaryotic species possess more than one P1 or P2 protein forms, such as *Saccharomyces cerevisiae* that contains two: P1α/P1β and P2α/P2β (2,3). The last 13 amino acids of the C-terminal domain (CTD) in five components of the pentamer, which mediate stalk activity during protein synthesis, are identical and are almost totally conserved in all eukaryotic species. The presence of a functional CTD in P0 is a key differential feature of the eukaryotic stalk, rendering the acidic proteins P1 and P2 non-essential for translation (4). In contrast, the prokaryotic P1/P2 counterpart, the L7/L12 protein, is required for protein synthesis (5), as the L10 protein lacks the corresponding functional CTD.

The eukaryotic stalk is a dynamic structure, whereby there is considerable exchange of P1/P2 proteins between the ribosome and a cytoplasmic pool of free proteins (6–8), and ribosomes either totally or partially lacking these acidic proteins exist in the cell (9). As stalk stability is essential for its proper functioning during translation, the aforementioned exchange implies that the stalk

structure undergoes cyclical conformational changes that affect the interaction of P0 with the P1/P2 heterodimers, thereby facilitating the release of the acidic proteins.

The CTD of the stalk acidic proteins exhibits a significant mobility, which has hindered the elucidation of the crystal structure of this ribosomal domain. Lately, a crystal structure of the prokaryotic stalk core was generated by removing most of the mobile region of the acidic proteins (10,11). Moreover, homology modelling has provided some insight into the structure of a large portion of the eukaryotic stalk (12,13), although direct experimental data remain elusive. Nonetheless, notable progress has been made in characterizing the interactions between eukaryotic stalk components, due to the use of a range of biochemical and biophysical approaches (14–25). By analysing the P0–P1/P2 interactions, P1/P2 heterodimers were seen to bind to specific contiguous sites in P0 (20,26,27). In wild-type *S. cerevisiae*, it has been reported that the first site (site A) lies between amino acids 199–230 in P0 and it binds the P1α/P2β heterodimers, while the P1β/P2α dimer mainly associates with site B lying between amino acids 231–258 (20). The size of the P0 and P1/P2 regions involved in these interactions (20,28), together with the information from homology modelling studies (12,13) and low resolution biophysical analyses of the complex (22), strongly suggest that the association of the acidic proteins with the core stalk protein is notably more complex in eukaryotes than in bacteria (10) or archaea (11). This complexity increases the potential of the stalk structure to undergo induced cyclical conformational changes, which probably mediate P1/P2 exchange. Moreover, small changes at the NTD can drastically affect the affinity of the acidic proteins for P0 (13).

While the need for two significantly different types of acidic proteins in eukaryotes (P1 and P2) remains unexplained, experimental evidence suggests that they might play distinct roles (29). The presence of multiple forms of P1 and P2 in some lower organisms remains equally perplexing, although they may represent an evolutionary response to environmental challenges to which higher organisms are not exposed. The four acidic proteins in *S. cerevisiae* appear to play distinct roles in standard laboratory conditions, and initial studies using stalk deletion mutants suggested that proteins P1α and P2β are more important for cell growth than P1β or P2α (30). A recently described stalk assembly model attributed a leading role to the P1α/P2β pair, proposed to be the first heterodimer to bind to site A of P0. The P1β/P2α pair would play a subsidiary function, subsequently binding to site B (20,24). The model implies a clear specificity of the P0 sites A and B for the corresponding P1/P2 couples, and suggests the formation of stable heterodimers prior to binding, at least in the case of P1α/P2β. However, evidence obtained from yeast strains lacking some of the stalk components suggests alternative combinations. *Saccharomyces cerevisiae* strains D47 and D56, which express only the 'non-canonical' P1α/P2α and P1β/P2β pairs, respectively, contain ribosomes with a functional stalk that carry the corresponding non-standard heterodimers (30). This observation suggests that the

12-kDa proteins can be assembled in the yeast stalk in different ways, depending on cell conditions, raising a number of questions regarding the specificity of the interactions between the distinct stalk components.

We performed a comparative detailed analysis of *S. cerevisiae* strains that individually express each of the four possible P1/P2 pairs, and quantified the structural and functional peculiarities of the corresponding ribosomal stalks, with a view to obtaining a better understanding of the function and assembly of this essential ribosomal domain.

MATERIALS AND METHODS

Organisms and growth conditions

Escherichia coli DH5α, grown in LB medium at 37°C, was used to propagate and maintain the plasmids. Bacterial transformations were performed as previously described (31). The *S. cerevisiae* strains used are listed in Supplementary Table S1, and they were grown at 30°C in YPD or SC medium with the appropriate metabolic requirements until mid-log phase. Yeast transformation was carried out using the lithium acetate method (32).

Plasmids

A series of plasmids encoding truncated P0 proteins that lack the P1/P2 binding sites A and B were constructed (Supplementary Table S2). The nucleotides encoding either site A (amino acids 198–230), site B (amino acids 230–258) or both sites together (amino acids 198–258) were removed from the *RPP0* gene encoded in the plasmid BS-P0 (33). The constructs were generated by an overlapping PCR strategy (Quickchange II site-directed mutagenesis kit: Stratagene) using BS-P0 as the template and the appropriate oligonucleotide primers (Supplementary Table S3). The *RPP0* gene was removed from BS-P0 as a BamHI–XhoI DNA fragment of 2.8–2.6 kbp, depending on the specific mutations incorporated, and inserted into either the BamHI–SalI sites of pFL36(LEU2) or the EcoRI–SalI sites of pFL37(HIS3), pFL38(URA3) and pFL39(TRP1) (34), depending on the genetic markers available in the strain to be transformed.

Cell fractionation and ribosome preparation

Total cell extracts and high-salt washed ribosomes from *S. cerevisiae* were prepared as summarized in the Supplementary Methods (28).

Analysis of polysomes and ribosomal subunits on sucrose gradients

Polysome profiles were prepared following standard methods, as summarized in the Supplementary Methods. In the ribosome dissociation experiments, the KCl concentration in cytoplasmic extracts was increased to 0.5 M (high salt-washed), and, the ribosomal subunits were resolved by centrifugation in a SW40Ti rotor at 39 000 rpm for 3 h 45 min at 4°C on a 10–30% (w/v) sucrose gradient in 15 mM Tris–HCl pH 7.4, 500 mM KCl and 5 mM $MgCl_2$.

Protein analysis

Ribosomal proteins were analysed by 15% SDS–PAGE or by isoelectrofocusing in 5% polyacrylamide gels over a pH range of 2.0–5.0 (35). The proteins were detected by Western blot using specific monoclonal antibodies against yeast stalk P proteins (36), or by silver staining. Proteins from total cell extracts were resolved by 2D polyacrylamide gel electrophoresis as described previously (37).

RESULTS

Phenotypic analysis of *S. cerevisiae* strains that contain a single P1/P2 heterodimer in the ribosomal stalk

Wild-type *S. cerevisiae* contains four acidic P proteins, P1α, P1β, P2α and P2β, which form two distinct P1/P2 heterodimers. Mutant strains carrying only one of the four possible P1/P2 pairs have been previously obtained (30), the strains D46, D47, D56 and D57 carrying the acidic protein couples P1α/P2β, P1β/P2β, P1α/P2α and P1β/P2α, respectively. The absence of one of the P1/P2 pairs does not appear to be particularly deleterious to the cell, although the effect varies depending on the specific proteins missing. Thus, in liquid rich medium at 30°C, the absence of P1β and P2α (strain D46) results in a 30% increase in doubling time, which increases to 70% in the other three double-disrupted strains (30). In addition, we analysed the response of these four mutants to temperature and osmotic stress, growing the strains on rich medium agar plates at 20, 30 and 37°C, in the presence or absence of 0.3 M NaCl (Figure 1). The growth effects were less noticeable on agar plates at 30°C, and they were almost undetectable at 37°C. However, the differences in growth rate were strongly accentuated at 20°C. Particularly strains D47 and D56, which contain the non-canonical heterodimers P1β/P2β and P1α/P2α, respectively, displayed a clear cold-sensitive phenotype, which was enhanced in the presence of NaCl.

Protein synthesis inhibitor sensitivity of stalk mutants

The response of the strains to sordarin and cycloheximide, two well-known eukaryotic translation inhibitors, was investigated by growing the yeast in rich liquid medium containing increasing drug concentrations (Supplementary Data). Both positive and negative changes in cell sensitivity (IC_{50}) to the drugs were detected depending on the proteins missing (Table 1). A significant decrease in IC_{50} was observed in the D56 and D57 strains treated with sordarin, while the drug sensitivity of the D46 and D47 strains did not change. In contrast, the response to cycloheximide was only significantly altered in strain D47, which contains the P1β/P2β dimer, resulting in an ~4-fold increase in the IC_{50}.

Effect of stalk composition on subunit association and polysome formation

Cell extracts from the parental strain and from the four stalk mutants were resolved on sucrose gradients (Figure 2). Both the number of polysome peaks and the
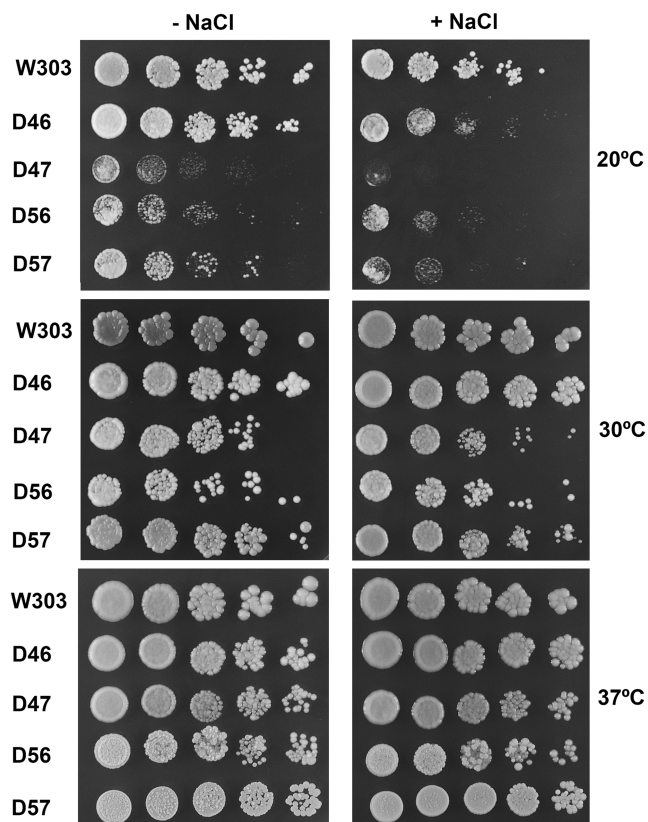

Figure 1. Response of *S. cerevisiae* stalk mutants to temperature and osmotic stress. Serial dilutions of cells from the four double mutants (D46, D47, D56 and D57), and of the parental W303 strain, were grown for 4 days on YEPD agar plates in the presence or absence of 0.3 M NaCl at 20, 30 or 37°C.

Table 1. Sensitivity of ribosomal stalk mutants to translation inhibitors

Strain	Stalk proteins present	Cycloheximide[a] IC_{50} μg/ml	Sordarin[a] IC_{50} μg/ml
W303	P1α, P1β, P2α, P2β	0.12 (1.00)	0.33 (1.00)
D46	P1α, P2β	0.10 (0.83)	0.34 (1.02)
D47	P1β, P2β	0.40 (3.40)	0.32 (0.97)
D56	P1α, P2α	0.14 (1.16)	0.19 (0.57)
D57	P1β, P2α	0.10 (0.82)	0.10 (0.30)

[a]IC_{50} values relative to W303 in brackets.

total amount of polysomes were lower in the mutants, although these effects were less pronounced in the D46 strain that exhibits a faster growth rate, and they were more evident in the slower growing mutants.

The presence of halfmers and an increase in the free subunit peaks was detected in the four mutant extracts, particularly in strains carrying non-canonical heterodimers (i.e. D47 and D56). The presence of halfmers in the polysome profiles may reflect a decrease in free 60S subunits in the cell, usually due to defective ribosome synthesis, or a decrease in the interactions between the ribosomal subunits that leads to the release of a fraction of the large subunits from polysomes during centrifugation.
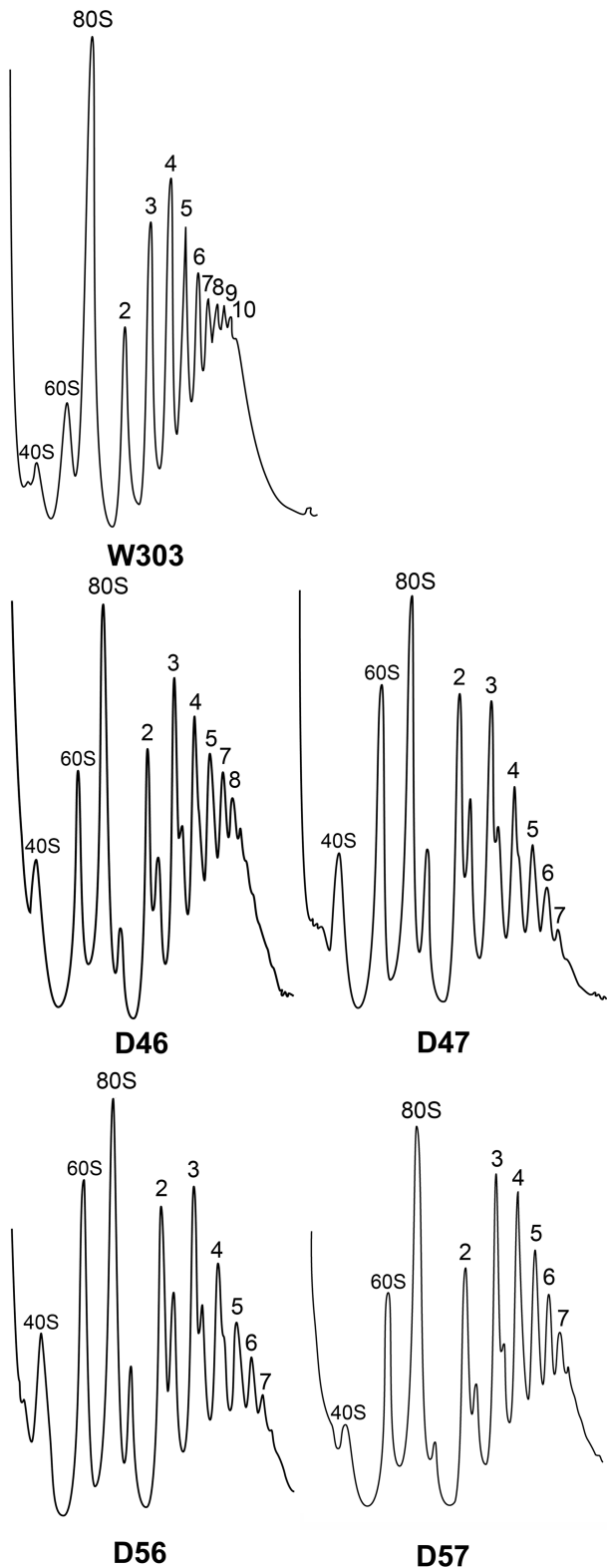
Figure 2. Total cell extracts from the strains indicated grown to mid-logarithmic phase (OD$_{600}$ = ~0.5), were resolved in 10–50% sucrose gradients under the conditions described in 'Materials and Methods' section, and the A$_{260}$ of the fractions was measured. The position of the 40S subunits, 60S subunits, 80S monosomes and polysomes containing increasing numbers of ribosomes is indicated.
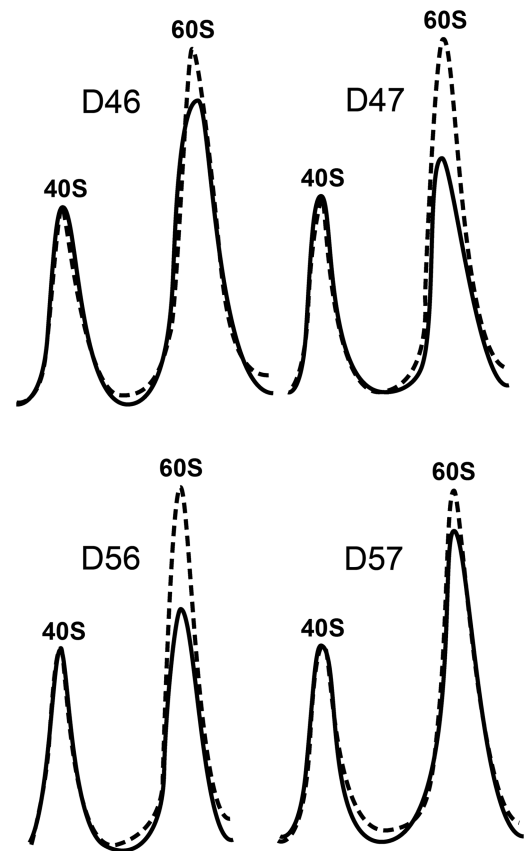

Figure 3. Total amount of ribosomal subunits in cell extracts resolved by sucrose gradients in dissociating conditions. The A$_{260}$ profile of the mutant extracts (continuous lines) is superimposed upon that of the parental W303 strain (discontinuous line). The position of the 40S and 60S subunits is indicated.

Calculating the overall 40S/60S ratio of the cell can help identify which of these two mechanisms is implicated. Thus, the total amount of each subunit was estimated by analysing cell extracts under ribosomal dissociation conditions, revealing a reduction in the amount of 60S subunits in all the mutant strains and particularly, in D47 and D56 (Figure 3).

Protein expression in ribosomal stalk mutants

The overall protein expression in each of the mutant strains was determined by 2D gel electrophoresis (Figure 4), revealing clear differences between the four strains. The relative intensity of many spots differed between mutants, and more significantly, several spots were observed in some mutant strains but not others. Hence, it would appear that the composition of the ribosomal stalk determines, either directly or indirectly, the efficiency of translation of some mRNAs, as previously shown in a mutant strain lacking P1/P2 proteins in the ribosome (4).

Analysis of ribosomal stalks in *S. cerevisiae* double mutants

In each of the four mutant strains the acidic proteins present in the ribosomes purified by a standard high-salt
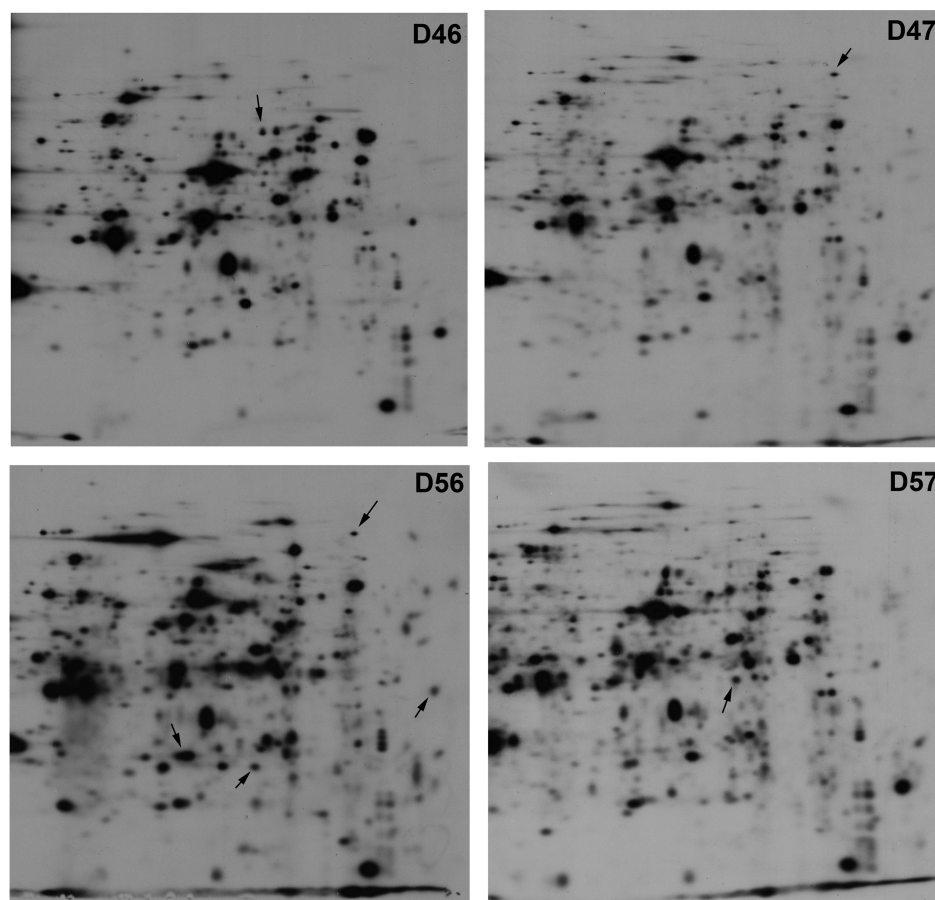
Figure 4. 2D electrophoresis of total extracts from mutant strains D46, D47, D56 and D57. Some of the differential spots that appear in one sample but not in others are indicated.

washing protocol were estimated in immunoblots probed with specific monoclonal antibodies against proteins P0, P1β, P2α and P2β. The ratio of each protein to P0 was comparable in the ribosomes of each mutant strain and the parental W303 strain (Figure 5). Although an equivalent monoclonal antibody to P1α was not available, the results from its partner protein P2β strongly indicated that this protein was also present in comparable proportions in all strains that express it.

These results indicate that the four possible P1/P2 heterodimers, including P1α/P2α and P1β/P2β, exhibit the same degree of ribosomal binding, suggesting that the specificity of P0 binding sites is not particularly stringent under the experimental conditions used.

Ribosomal stalk components in strains expressing truncated P0 proteins that lack P1/P2 binding sites

To better understand how non-canonical P1/P2 heterodimers are assembled in stalks, plasmids were constructed encoding different P0-truncated derivatives, similar to those described previously (20). Accordingly, the protein P0ΔA lacks site A (amino acids 198–230), protein P0ΔB lacks site B (amino acids 230–258) and the P0ΔAB protein lacks both sites (amino acids 198–258). The three truncated proteins, as well as the native P0, were expressed in conditional P0 null strains

(W303dGP0, D46dGP0, D47dGP0, D56dGP0 and D57dGP0) that only express the plasmid-encoded protein when grown in glucose medium (33). Total extracts from the transformed strains grown in glucose were resolved by SDS–PAGE and the proteins were detected in immunoblots probed with antibodies specific to the CTD of eukaryotic stalk proteins. There was an expected decrease in the size of the truncated P0 proteins (Supplementary Figure S1) and interestingly, the P1/P2 proteins were absent from the total extracts of double-disrupted mutants expressing the P0ΔAB form. Moreover, these proteins were notably reduced in the W303dGP0/P0ΔAB strain.

The effect of expressing a truncated P0 on the growth rate of double-disrupted mutants was estimated by serial dilution tests using rich medium agar plates. The presence of either P0ΔA or P0ΔB had no significant effect on the growth of strains D46 and D57, when compared with the strains expressing the wild-type P0 (Supplementary Figure S2).

Deletion of P0 acidic protein binding sites: effects on stalk composition

Ribosomes from the transformed strains, purified and high-salt washed according to standard protocols, were analysed by isoelectrofocusing in a pH range of 2.0–5.0
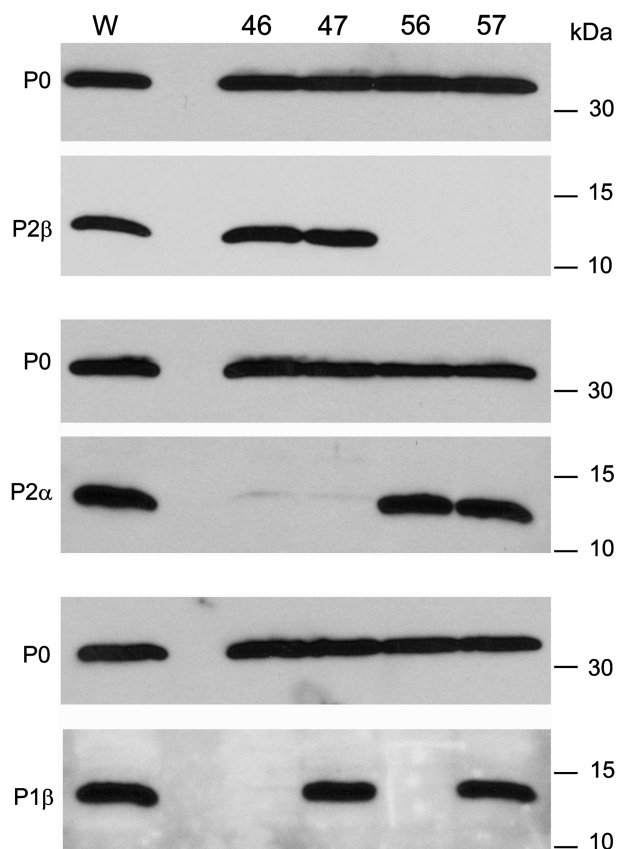
Figure 5. Analysis of stalk proteins in purified ribosomes from mutant strains. Ribosomes purified from the indicated mutant strains and the parental W303 (W) strain were resolved by 15% SDS–PAGE and the indicated proteins were detected in immunoblots probed with specific monoclonal antibodies.

to resolve the different P1/P2 forms. The absence of site A in the parental W303dGP0/P0ΔA strain yielded ribosomes that almost exclusively contained P1β and P2α proteins (Figure 6A). In contrast, the four acidic proteins were present in comparable amounts in ribosomes from W303dGP0/P0ΔB, which only carries site A. This unexpectedly low specificity of site A was confirmed in cells from two different transformation experiments (ΔP0ΔB1 and ΔP0ΔB2 in Figure 6A). As expected, elimination of both binding sites resulted in the absence of acidic proteins in ribosome from W303dGP0/P0ΔAB.

When the truncated proteins were expressed in strain D46dGP0 (that contains P1α and P2β alone), both proteins were detected in normal amounts in ribosomes containing site A (D46dGP0/P0ΔB), as was expected. However, both proteins were also notably present in D46dGP0/P0ΔA ribosomes that only contained site B (Figure 6B). Similarly, in D57 the amount of P1β and P2α was greater when the P0 carried only site B (D57dGP0/P0ΔA), although both proteins also bound to site A in D57dGP0/P0ΔB ribosomes (Figure 6D). In both these strains, the expression of P0ΔAB eliminates the acidic proteins from the cell (Supplementary Figure S1) and consequently from the ribosome (Figure 6B and D).

In strains D47 and D56 (which express the non-canonical heterodimers P1β/P2 and P1α/P2α, respectively), comparable amounts of acidic proteins were bound to the ribosomes regardless of the P0 protein present, except in the case of P0ΔAB that totally prevented the accumulation of all P1/P2 proteins in the cell (Figure 6C and E).

DISCUSSION

The stalk is essential for proper ribosomal function, and stalk defective ribosomes have a partially reduced translational efficiency (4). It is, therefore, not surprising that alteration in the stalk composition affects protein synthesis and consequently cell growth in eukaryotes. These effects vary depending on the proteins missing from the stalk. Thus, the absence of proteins P1β and P2α in strain D46 provoked a slight reduction in growth as compared to wild-type controls. This reduction was much more pronounced in D57, which lacks P1α and P2β, and particularly in D47 and D56, which carry the non-canonical pairs P1β/P2β and P1α/P2α, respectively. The growth effect was minimal at 30°C and almost undetectable at 37°C, although it was drastic at 20°C indicating a clear cold-sensitive phenotype. These results emphasize the different roles of the four acidic P proteins in stalk activity.

The changes in cell growth were largely paralleled by the polysome profiles of mutant extracts. Thus, the polysome area and the number of polysome peaks were greater in extracts from D46, the fastest growing mutant. Halfmers were also evident in these profiles and there was an increase in free ribosomal subunits in mutant extracts, particularly in D47, D56 and D57. Moreover, estimation of the total amount of 40S and 60S in extracts under dissociating conditions indicates a specific reduction in large subunits in the presence of a defective ribosomal stalk. This effect was more notable in D47 and D56, which express the non-canonical heterodimers P1β/P2β and P1α/P2α, respectively. Although it cannot be fully ruled out, it is unlikely that this reduction is due to the targeting of mature subunits carrying an altered stalk for degradation, as these subunits are functional and are efficiently incorporated into polysomes. The diminished expression of the 60S subunit is more likely to be due to alterations in the assembly pathway induced by the unusual P1/P2 expression in the mutants, which induces the generation of a fraction of defective pre-ribosomal particles that are degraded before maturing (38). The cold-sensitive phenotype of these mutants is in fact a typical feature of cells with a deregulated ribosomal assembly process (39). Thus, the P1/P2 proteins would appear to play a role in the 60S assembly process.

The functional differences displayed by the stalk mutants result in an alteration of the overall pattern of protein expressed. The total absence of P1/P2 proteins was shown to affect the translation of specific mRNAs, leading to the proposal that these proteins can regulate translation acting as modulators of ribosomal function (2,4). The present results indicate that ribosome carrying unusual
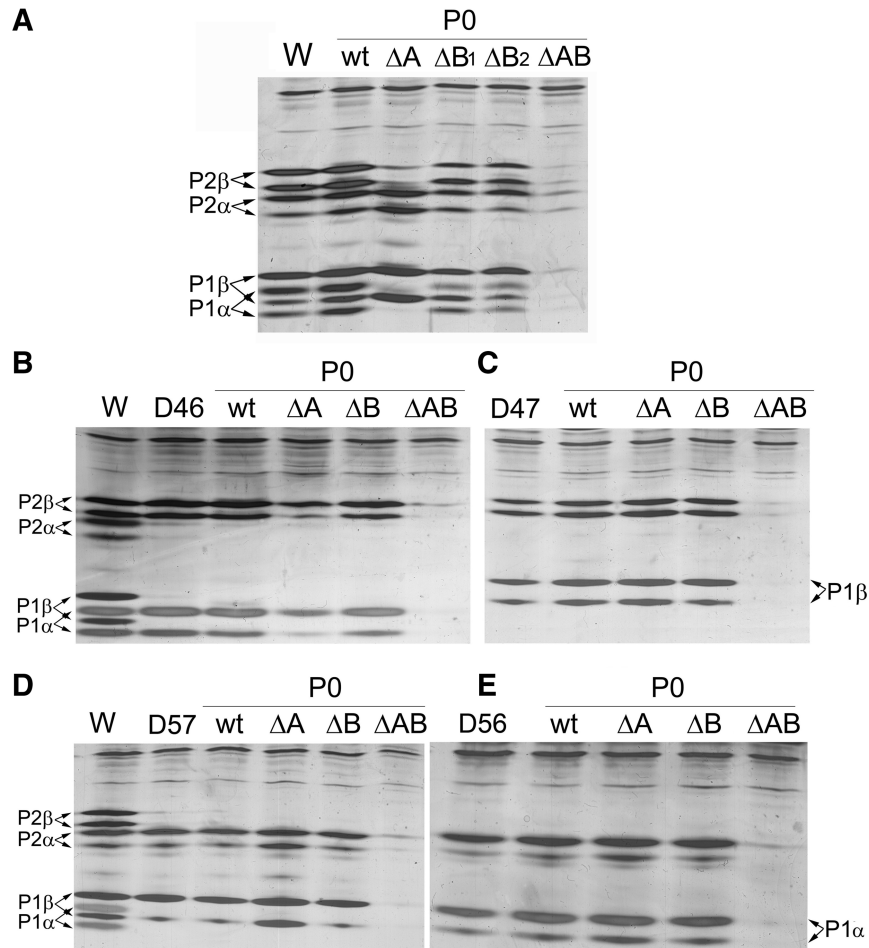
Figure 6. Ribosomal stalk composition of mutant yeast strains carrying truncated P0 proteins. Ribosomes were purified from the wild-type W303 strain (**A**), from the D46 (**B**) and D57 (**D**) mutants containing canonical heterodimers, and from the D47 (**C**) and D56 (**E**) mutants containing non-canonical heterodimers, expressing either wild-type P0 (P0wt) or truncated P0 proteins ΔA, ΔB and ΔAB (see Supplementary Figure S1). The ribosomes were resolved by isoelectrofocusing in a pH range of 2.0–5.0 and the proteins were detected by silver staining. As a control, purified ribosomes from the respective non-transformed strain were included in the corresponding gels. In gel A, ribosomes from two clones from two different transformations of W303dGP0 with protein P0-B (ΔB$_1$ and ΔB$_2$) were included. The upper and lower bands correspond to the phosphorylated and non-phosphorylated forms of each protein.

ribosomal stalk compositions can also participate in the proposed regulatory process.

The sensitivity to two well-known inhibitors of translation elongation, cycloheximide and sordarin, was also influenced in a distinct manner by the stalk alterations of each mutant strain. While some mutants remained practically unaffected, others exhibited significant alterations in their IC$_{50}$ values. Indeed, D47 was more resistant to cycloheximide while D56 and D57 were significantly more sensitive to sordarin than the parental strains. Given the poor understanding of the molecular mechanism of cycloheximide inhibition at the molecular level, the role of different ribosomal stalk components in drug activity remains unclear. In contrast, the well-documented mode of action of sordarin may help us interpret our results. GTP hydrolysis provokes conformational changes in the elongation factor, which generate a signal required for the completion of translocation. EF2-bound sordarin blocks the transmission of this signal, as revealed by crystal structure analysis of EF2-bound drug (40) and

Cryo-EM analysis of the 80S–EF2–sordarin complex (41). Mutations in the ribosomal stalk protein P0 can induce resistance to sordarin without blocking drug binding, which takes place far from the stalk (42,43). These mutations probably restore signal transmission in the presence of the drug, thereby implicating P0 in this process. Our results indicate that P1α and particularly P2β are relevant to this process, but not P1β and P2α, as mutants lacking the P2 protein (D56) or both proteins (D57) were notably more sensitive to sordarin.

The specificity observed for the P1/P2 binding sites of the P0 protein was particularly interesting. First, the non-canonical couples P1α/P2α and P1β/P2β were detected in purified ribosomes from strains D56 and D47 at comparable proportions to the canonical pairs in strains D46 and D57 (Figure 5), suggesting that site specificity is not particularly stringent regarding the heterodimer composition. This conclusion was confirmed by expressing a truncated P0 lacking one P1/P2 binding site in the double mutants expressing only one of the four

possible P1/P2 pairs. In all cases, the P1 and P2 proteins expressed bound to the ribosome in roughly stoichiometric amounts, irrespective of the P0 binding site. Taken together, these results suggest that the P0 sites A and B can bind any P1/P2 combination with comparable efficiency. However, the response of the deletion of the P0 binding sites observed in W303 strain, which contains the four acidic proteins, was different. In this case, site A exhibits little specificity and it binds the four proteins in similar proportions when site B is deleted, while site B shows strong specificity for P1β/P2α when site A is deleted. This conclusion is confirmed by the reduced levels of P1α/P2β observed in strain D46ΔA in which only site B is present.

These results do not support the stalk assembly pathway involving binding at site A prior to site B previously proposed (24). As this model was based on data from fully assembled ribosomes, it is difficult to deduce directly how the stalk was actually assembled. Moreover, the results indicated a stabilization of the structure upon formation of the stalk pentamer (24), implying that significant changes occur in the preceding complexes during the assembly process. The differential specificity of P0 sites reported here suggests an alternative assembly pathway (Supplementary Figure S3). We propose that stalk assembly begins with the binding of P1β/P2α to site B. Positive coupling of both binding sites then facilitates the subsequent interaction of P1α/P2β at site A, which is also likely to be promoted by the increased local concentration of these heterodimers resulting from the prior binding of P1β/P2α at site B. The occupation of both sites stabilizes the assembled P1α/P2β–P0–P1β/P2α pentamer, as reported previously (24). However, the weak specificity of site A also permits a small proportion of P1β/P2α heterodimers to bind to this site and form a P0–(P1β/P2α)$_2$ complex (see Supplementary Figure S3). Accordingly, experimental evidence demonstrated that in the cell, ribosomes may carry unusual P1/P2 heterodimer combinations. The formation of P protein homodimers has been described in ribosome cross-linking studies (29), demonstrating that two copies of the same heterodimer may exist in the stalk. Moreover, fluorescence correlation spectroscopy using strains expressing GFP-labelled stalk components has shown that cells may contain a small fraction of ribosomes carrying unexpected amounts of P proteins incompatible with the standard stalk composition (44).

Our current understanding of stalk assembly is limited. Where and when P1/P2 proteins bind to P0, and the possible role of helper proteins in this process, remain unclear (45–47). As such, any proposed assembly model should be considered as a working hypothesis that awaits further experimental support. Nevertheless, the data available indicate that in the yeast wild-type ribosome, P1α/P2β dimers are predominantly found at site A and P1β/P2α at site B, although a fraction of particles may contain alternative P1/P2 heterodimer distributions. Thus, stalk assembly mechanism appears to be able to generate a variable level of stalk heterogeneity in the ribosomal population. This heterogeneity is likely to be important for the proposed regulatory functions of the stalk (2)

and it is probably controlled by the cell, which regulates the expression of different P1 and P2 proteins, thereby affecting the proportion of distinct P1/P2 pairs in the cytoplasm (48). In connection with this issue, the drastic decrease in P1/P2 protein accumulation in the presence of P0ΔAB (Supplementary Figure S1) indicates a possible involvement of P0 in the control of P1/P2 expression, which require further investigation.

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