Assembly of *Saccharomyces cerevisiae* Ribosomal Stalk: Binding of P1 Proteins Is Required for the Interaction of P2 Proteins†

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ABSTRACT: The yeast ribosomal stalk is formed by a protein pentamer made of the 38 kDa P0 and four 12 kDa acidic P1/P2. The interaction of recombinant acidic proteins P1α and P2*β* with ribosomes from *Saccharomyces cerevisiae* D4567, lacking all the 12 kDa stalk components, has been used to study the in vitro assembly of this important ribosomal structure. Stimulation of the ribosome activity was obtained by incubating simultaneously the particles with both proteins, which were nonphosphorylated initially and remained unmodified afterward. The N-terminus state, free or blocked, did not affect either the binding or reactivating activity of both proteins. Independent incubation with each protein did not affect the activity of the particles, however, protein P2*β* alone was unable to bind the ribosome whereas P1α could. The binding of P1α alone is a saturable process in acidic-protein-deficient ribosomes and does not take place in complete wild-type particles. Binding of P1 proteins in the absence of P2 proteins takes also place in vivo, when protein P1*β* is overexpressed in *S. cerevisiae*. In contrast, protein P2*β* is not detected in the ribosome in the P1-deficient D67 strain despite being accumulated in the cytoplasm. The results confirm that neither phosphorylation nor N-terminal blocking of the 12 kDa acidic proteins is required for the assembly and function of the yeast stalk. More importantly, and regardless of the involvement of other elements, they indicate that stalk assembling is a coordinated process, in which P1 proteins would provide a ribosomal anchorage to P2 proteins, and P2 components would confer functionality to the complex.

The ribosomal stalk has an essential role in translation, participating in the interaction of the elongation factors with the ribosome (*1*). In addition, the recent characterization of stalk protein mutations conferring resistance to eukaryotic elongation factor 2 inhibitors indicates that the stalk is also involved in the translocation mechanism (*2*, *3*).

The stalk is formed by a pentameric protein complex, which in bacteria is made of one copy of protein L10 and two dimers of the acidic protein L7/L12. In mammals, this pentameric complex is formed by P0, the 38 kDa L10 protein-equivalent, and two dimers of the 12 kDa acidic proteins P1/P2 (*4*, *5*). In lower eukaryotes and plants there are several forms of acidic proteins (see ref *6* for a review). Two proteins of the P1-type, P1α and P1*β*, and two of the P2-type, P2α and P2*β*, have been reported in *Saccharomyces cerevisiae* cells (*7*). As in mammals, five proteins seem to form the yeast stalk in exponentially growing cells (*8*). However, contrary to mammals, the yeast 12 kDa proteins are not present as dimers, but there seems to be only one molecule of each one of the four acidic proteins per ribosome (*9*). Nevertheless, gene disruption studies have shown that the presence of at least one protein of each type, P1 and P2, is required to form the stalk (*10*).

The eukaryotic stalk is much less stable than the bacterial one. The amount of 12 kDa proteins in the ribosome is not constant, and it varies with the yeast ribosome preparation depending on the metabolic state of the cell (*8*). Moreover, a cytoplasmic pool of the stalk components has been found, and the proteins in this pool are exchanged with the ribosome-bound polypeptides during protein synthesis (*11*−*13*). There are experimental data suggesting that due to this instability, the stalk is probably part of a translation control process that, by regulating the ribosome activity could modulate the expression of some specific proteins (*6*).

The eukaryotic stalk components are found phosphorylated in the ribosome but not in the cytoplasmic pool (*14*). In addition, it has been shown that the in vitro treatment of proteins P1 and P2 with alkaline phosphatase abolishes the capacity of the proteins to bind to P-protein deficient ribosomes and to reconstitute the activity of the particles (*15*, *16*). These data suggested that P protein phosphorylation plays a role in the interaction of the acidic proteins with the ribosome. However, recent results have clearly indicated that mutation of the phosphorylated site in each one of the *S. cerevisiae* stalk components does not affect their capacity to individually interact with the ribosome in the cell, although it has some effect on the translation process (*17*, *18*). There are, therefore, some apparent contradictions between the in vitro and the in vivo data with respect to the requirements for the formation of the yeast stalk.

Despite the relevance that the eukaryotic stalk dynamics seems to have in the translation, the information available on the assembly process is quite scarce. Only one report on

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the in vitro reconstitution of the rat liver GTPase domain from its RNA and protein components is available (*19*). Using recombinant stalk proteins and ribosomes lacking the 12 kDa acidic proteins from P1/P2 disrupted strains (*10*, *20*), the interaction of the acidic P proteins with the ribosome has been studied. The results indicate that the assembly of the acidic proteins to form the ribosomal stalk is an ordered process that probably is not identical in the cell and in the test tube.

EXPERIMENTAL PROCEDURES

Strains and Growth Media. S. cerevisiae W303-1b [α, *leu2-3,112, trp1-1, ura3-1, his 3-11,15, ade2-1, can1-100*] was used as control strain. *S. cerevisiae* D45 [α; *leu2-3, 112, trp1-1, RPYP2*α::*URA3, RPYP2β*::*HIS3*], *S. cerevisiae* D67 [α; *ura3-1, his3-11,15, rpYP1*α*::LEU2, rpYP1β(L44′)::TRP1*] (*10*) and *S. cerevisiae* D4567 (α; *RPYP1*α*::LEU2, RPYP1β*::TRP1, RPYP2α::*URA3, RPYP2β*::*HIS3, can1-100*) (*20*). Yeasts were grown in either YEPD (1% yeast extract/2% peptone/2% glucose) or YNBD (0.67% amino acid-free yeast nitrogen base/2% glucose).

Escherichia coli DH5α, grown in LB medium (1.0% bacto-tryptone/0.5% yeast extract/0.5% NaCl) was used for transformation and propagation of plasmids. *E. coli* BL21(DE3)plac (*21*) grown in Terrific broth (*22*) was used to express the recombinant proteins.

Cell Transformation. E. coli cells were transformed according to (*23*). *S. cerevisiae* transformations were performed by the Lithium acetate method (*24*).

Recombinant DNA Techniques. Restriction endonucleases, T4 DNA ligase, Klenow DNA polymerase I fragment, and other enzymes were purchased from either Boehringer Mannheim (Germany), New England Biolabs, or Amersham (U.K).

DNA preparation, restriction enzyme digestions, agarose gel electrophoresis, ligation of DNA fragments, Southern blots, etc. were carried out according to standard techniques (*22*). DNA was sequenced by the dideoxy chain termination method (*25*) using universal primers and complementary oligonucleotides.

Plasmids. YEp356Trp/P1α, containing the *RPP1*α gene, was prepared by subcloning the gene as a *Bam*HI−*Hin*dIII 1. 97 kbp fragment from plasmid BS-L47 (*17*) into the corresponding sites in the MCS[1] of the multicopy vector YEp356 (*26*). Afterward, the *TRP1* marker was introduced as a *Bgl*II−*Bgl*II 0.8 kbp fragment into the *Bam*HI site of the previous construct.

YEp13/P1*β* was constructed by subcloning a *Hin*dIII−*Hin*dIII 1.98 kbp fragment from plasmid pMRH46 (*27*), containing the *RPP1β* gene, between two *Hin*dIII sites in position 0.03 and 10.60 in YEp13 (*28*).

Expression and Purification of Recombinant Proteins. The P2*β* protein was expressed from the *RRP2β* gene subcloned in the pT7-7 vector (*21*, *29*) and purified as described previously (*30*). Protein P1α and the truncated form of P2*β* (P2β^t), lacking the last 38 amino acids, were also expressed from the genes subcloned in pT7-7 and purified by similar procedures (*31*).

Protein Analysis. SDS polyacrylamide gel electrophoresis and isoelectrofocusing of proteins were performed as described (*17*) except that whole ribosomes were used directly as sample. Ribosomes (0.5 mg) were incubated with 10 *μ*g of RNAsa A for 30 min at 30 °C, lyophilized and resuspended in the sample buffer for electrophoresis.

Mass spectrometry measurements were done on an electrospray mass spectrometer (Peptide and Nucleotide Laboratory, Dept. of Organic Chemistry, Universidad de Barcelona). Western blot and ELISA assays were carried out using a set of previously characterized specific monoclonal antibodies (*32*). The four native ribosomal acidic proteins from *S. cerevisiae* ribosomes (SP fractions), were prepared as reported earlier (*33*). Estimation of total protein in cell extracts was carried out according to ref *34*.

Cell Fractionation. Cells were broken with glass beads and the extracts were centrifuged for 15 min at 15 000 rpm in a Sorvall SS34 rotor to obtain an S-30 total extract fraction. Ribosomes and S-100 supernatants were prepared from the S-30 extracts by high-speed centrifugation at 100000*g* (*33*). Ribosomes were washed by centrifugation for 20 h at 45 000 rpm throughout a 20−40% sucrose gradient in 30 mM Tris-HCl, pH 7.4, 50 mM $MgCl_2$, 500 mM NH_4Cl, and 5 mM *β*-mercaptoethanol. The ribosomes recovered in the pellet were resuspended in 20 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 80 mM KCl, and 5 mM *β*-mercaptoethanol (buffer 1) and stored at −20 °C.

Reconstitution of Ribosomes. Ribosomes (0.75 nmol) were incubated with the appropriate amount of protein (1−10 molar excess) in buffer 1 at 37 °C for 30 min and then washed through a 20−40% sucrose gradient in buffer 1 as previously indicated. The particles were recovered by centrifugation, resuspended in buffer 1 and used for either activity test or isoelectrofocusing analysis.

Activity Tests: Polyphenylalanine Synthesis. The reaction was performed in 50 *μ*L samples containing 10 pmol of 80S ribosomes, 5 *μ*L of S-100, 0.5 mg/mL tRNA, 0.3 mg/mL polyuridylic acid, 40 mM [^{3}H]phenylalanine (120 cpm/pmol), 0.5 mM GTP, 1 mM ATP, 2 mM phosphocreatine and 40 mg/mL of creatine phosphokinase in 50 mM Tris-HCl, pH 7.6, 15 mM $MgCl_2$, 90 mM KCl, and 5 mM *β*-mercaptoethanol. After incubation at 30 °C for 30 min, samples were precipitated with 10% TCA, boiled for 10 min and filtered through glass fiber filters.

RESULTS

*Interaction of Recombinant P1*α *and P2β Proteins with the Ribosome.* The *S. cerevisiae* ribosomal stalk proteins P1α and P2*β*, as well as P2β^t, a derivative of P2*β* lacking the last 38 amino acids, were expressed in *E. coli* from pT7-7 plasmids and purified as previously described (*30*). Protein P2*β* is obtained from the bacterial extracts in two forms, the native polypeptide with an unblocked amino end, and P2β_f, its N-terminal formylated derivative (*30*). In contrast, P1α is recovered as a unique N-terminal-unblocked protein. The presence of a free amino group changes the mobility of P1α in the isoelectrofocusing gels to a position more basic than the native N-terminal-acetylated protein found in the yeast ribosomes (see below). It must be noted that unblocking of the P1-type protein amino end does not affect the interaction of the protein with the ribosome in the cell (*35*).

[1] Abbreviations: ELISA, enzyme-linked immunosorbent sandwich assay; MCS, multiple cloning site.

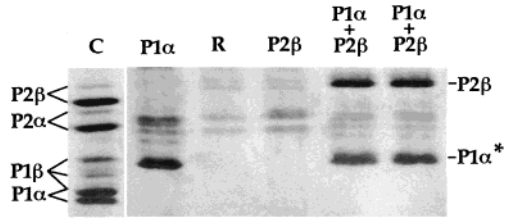

FIGURE 1: Isoelectrofocusing of reconstituted yeast ribosomes. Ribosomes from *S. cerevisiae* D4567 lacking all the stalk acidic proteins were incubated with recombinant proteins P1α, P2*β*, P1α + P2*β* as marked. After incubation in reconstitution conditions, the particles were centrifuged through a sucrose gradient and after treatment with RNase, directly applied to the gel. Ribosomes from strain D4567 incubated in the same conditions in the absence of recombinant proteins (R), as well as particles from the parental strain W303 (C), were included as controls. In all cases each protein was in a 4-fold molar excess over the ribosomes, except in the last right-hand lane, where they were in an 8-fold excess. The position of the proteins is marked in the figure. The lower band in each case corresponds to the phosphorylated form of the protein. P1α* indicates the position of the recombinant protein.

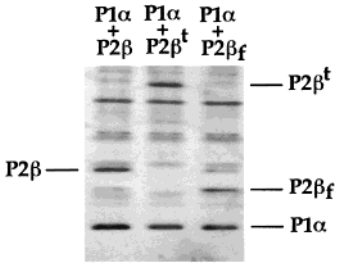

FIGURE 2: Reconstitution with different P2*β* proteins. Ribosomes were incubated in the presence of recombinant proteins as marked, in the conditions described in Figure 1. A 4-fold excess of recombinant proteins P1α, P2*β*, and P2β_f, and an 8-fold excess of P2β^t, were added in each case.

Although these proteins are found phosphorylated in the yeast ribosomes, they were obtained unmodified from the bacterial extracts as indicated by isoelectrofocusing of the purified preparations.

The recombinant proteins, either separately or simultaneously, were incubated with ribosomes lacking the four acidic P proteins obtained from *S. cerevisiae* D4567 (*20*) in reconstitution conditions. The ribosomes were afterward collected by centrifugation through a sucrose gradient and the acidic proteins in the particles were analyzed by isoelectrofocusing (Figure 1). Proteins P1α and P2*β* appear bound in its nonphosphorylated form when both were simultaneously incubated with the ribosome. The binding seems to be saturated when incubation is performed at around a 4-fold molar excess of proteins, and the amount of bound protein is similar in the reconstituted particles and in the control wild-type ribosomes. As predicted from the previous in vivo data (*10*), P2*β* is unable to bind alone to the ribosomes but protein P1α is present in the ribosome when incubated alone in the same conditions.

Interaction of Modified P2β Proteins. Similar reconstitution tests were performed using the two purified P2*β* derivatives, P2β_f, and P2β^t. Both proteins were able to bind to the ribosomes only in the presence of P1α (Figure 2). The removal of the highly acidic P2*β* carboxyl end causes a notable increase in protein pI that results in an important change in the position of the corresponding band in the isoelectrofocusing gel. In contrast, the formylated P2β_f has a lower pI and moves to a position below the native protein. The binding of P2β_f is not different from the one of the native protein; however, twice as much P2β^t was required to obtain a comparable binding (Figure 2).

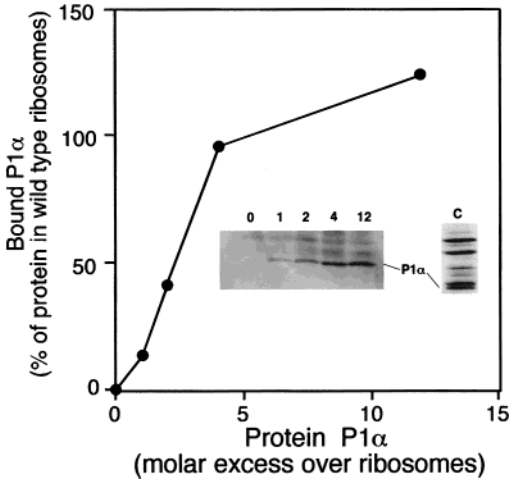

FIGURE 3: Binding of protein P1α to *S. cerevisiae* D4567 ribosomes. Increasing amounts of protein P1α were incubated in reconstitution conditions with ribosomes as indicated in Figure 1. Ribosomes were resolved by isoelectrofocusing (inset), and the band intensity estimated by densitometry using the protein in wild-type ribosomes (C) as a control. Numbers over the gel correspond to the molar excess of protein over ribosomes.

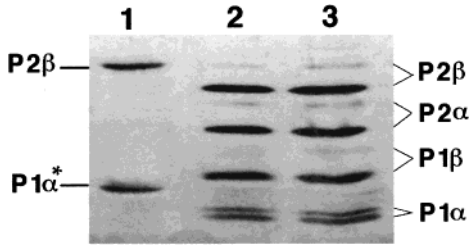

FIGURE 4: Binding of recombinant proteins to wild-type *S. cerevisiae* W303 ribosomes. Ribosomes from *S. cerevisiae* W303 were incubated with a 4-fold excess of proteins P1α and P2*β* (2) and processed as described in Figure 1. Ribosomes treated similarly in the absence of exogenous protein (3) as well as free recombinant proteins (1) were used as control. P1α* indicates the position of the recombinant protein.

Interaction of Protein P1α with Ribosomes. The binding of protein P1α alone to the ribosomes was unexpected, since the previous in vivo data seemed to indicate that the presence of one protein of each type, P1 and P2, is required for binding to the ribosome (*10*). To characterize more precisely this unanticipated interaction of protein P1α alone with the ribosome, its binding was measured as a function of the amount of protein in the reaction mixture. As is shown in Figure 3, the amount of protein associated to the ribosomes reaches a plateau, indicating that the binding is a saturable process. In addition, the amount of bound protein is similar to the amount of protein found in the wild-type ribosomes (Figure 3). Moreover, the lack of P1α binding to wild-type ribosomes (Figure 4) would indicate that an empty site in the ribosomal stalk is required for the interaction to take place. All these results strongly support the specificity of the P1α interaction with the ribosome.

Activity of the Reconstituted Ribosomes. The activity of the reconstituted particles was tested in an in vitro amino acid polymerizing system to check the functionality of the

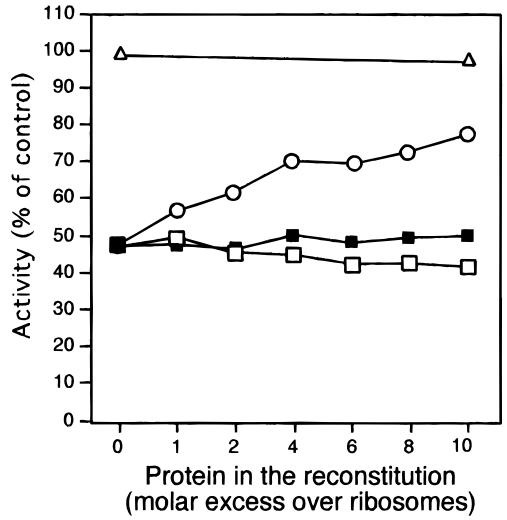

FIGURE 5: Polymerizing activity of reconstituted ribosomes. *S. cerevisiae* D4567 ribosomes were reconstituted in the presence of increasing amounts of recombinant P1α (□), P2*β* (■), and P1α + P2*β* (○), recovered by centrifugation and tested in a poly-Phe synthesis assay. The activity of ribosomes from *S. cerevisiae* W303-1b (△) in the absence of recombinant protein was taken as a control (100%). In all cases an extract (S100 fraction) from *S. cerevisiae* D4567 was used as a source of supernatant factors to avoid the presence of free acidic proteins in the reaction mixture.

bound proteins. Only the simultaneous binding of the two proteins can reactivate the activity of the particles to roughly 80% of the wild-type controls (Figure 5). This value is not far from the expected activity of particles, which still lack part of the stalk components, namely proteins P1*β* and P2α (*10*, *20*). As expected from the binding results, the incubation with P2*β* alone did not stimulate the activity of the ribosomes. Similarly, despite its binding capacity, protein P1α alone does not have a stimulatory effect, but even it causes a slight decrease in the ribosome activity.

Binding of 12 kDa P Proteins to Ribosomes in Vivo. Previous results have shown that ribosomes from *S. cerevisiae* D45 and D67, which express only acidic proteins of either the P1-type (D45) or P2-type (D67), exclusively contain protein P0 in the stalk (*10*). These results suggested that the presence in the cell of at least one acidic protein of each type was required to form the complex with P0 in the ribosome. The in vitro binding of P1α to ribosomes in the absence of P2 proteins seemed to contradict that conclusion and prompted a reexamination of the in vivo results.

An estimate of the 12 kDa proteins expressed in strains D45 and D67 was performed in total S-30 cell extracts by western blotting using a monoclonal antibody specific for the C-terminal of all the P proteins (*32*). It was found that the amount of 12 kDa acidic proteins in D67 (Figure 6, lane 5) was clearly reduced, but it was still comparable with that present in the parental W303 strain (Figure 6, lane 1). The presence of acidic proteins in the total D67 extracts, together with their absence in the respective ribosomes (*10*), confirms that P2 proteins are really unable to bind to the ribosome in the absence of P1 proteins, which are not expressed in this strain.

On the other hand, the absence of acidic proteins, previously reported in the D45 ribosome (*10*), might simply be due to the very low amount of these polypeptides present in the cell (Figure 6, lane 2). To examine this possibility

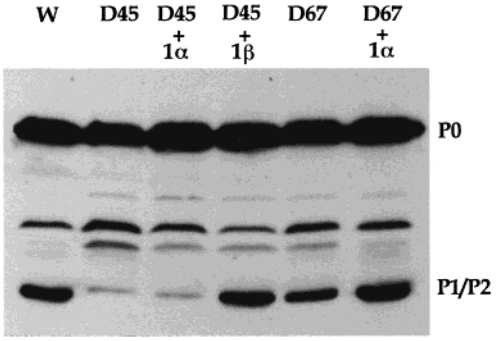

FIGURE 6: Estimation of 12 kDa acidic proteins in extracts from different yeast strains. Total cell extracts (S30 fraction) from *S. cerevisiae* D45 transformed with YEp356Trp/P1α (D45 + 1α), D45 transformed with YEp13-P1*β* (D45 + 1*β*), D67 (5), and D67 transformed with YEp356Trp/P1α (D67 + 1α) were resolved by SDS−PAGE. Extracts from *S. cerevisiae* W303 (W) were included as a control. Proteins were transferred to inmobilon membranes, and detected using a specific monoclonal to the conserved C-terminal domain of all acidic P proteins. The position of the different P proteins is marked.

Table 1: Estimation[a] of Protein P1*β* in Cellular Supernatant Fractions

	protein in extracts from *S. cerevisiae*		
protein	D45 μg/mg S100	D45/1*β*[b] μg/mg S100	W303 μg/mg S100
P1*β*	0.0192	0.833	0.072

[a] Total protein was estimated in S100 supernatant fractions (see Experimental Procedures) according to ref *34* and protein P1*β* by ELISA using specific monoclonal antibodies (*32*). [b] *S. cerevisiae* D45 transformed with plasmid YEp13/P1*β*.

and to test whether P1 proteins can actually bind alone to the ribosome in the cell, overexpression of their genes in *S. cerevisiae* D45 was attempted. The P1 genes, cloned in the multicopy plasmids YEp13/P1*β* and YEp356Trp/P1α, were used to transform this strain, and total S-30 extracts from the transformed cells were also resolved in SDS gels and are included in Figure 6.

An accumulation of acidic proteins was not detected in the YEp356Trp/P1α transformant, which showed the same weak 12 kDa band as did the nontransformed cells (Figure 6, lanes 2 and 3). Since YEp356Trp/P1α is functional, as shown by the increase of the acidic protein band detected in D67 transformed with this plasmid (Figure 6, lanes 5 and 6), it seems that P1α expression is tightly controlled in D45 cells impeding protein accumulation. There are experimental data indicating that this control is carried out by degradation of the free protein (G. Nusspaumer, M. Remacha and J. P. G. Ballesta, unpublished results).

In contrast, a notable increase of the acidic protein band was found in extracts from D45 transformed with YEp13/P1*β*. Although the amount of P1*β* is probably less than expected from the high number of plasmid copies and the amount of mRNA (not shown), the protein accumulates in the cell (Figure 6, lanes 2 and 4). Since the 12 kDa proteins are mainly bound to the ribosomes in wild-type cells, the accumulation is most manifest in the post-ribosomal supernatant where the amount of P1*β* is 10 times higher than in the parental W303-1b strain as estimated by ELISA using a specific monoclonal antibody (Table 1). Ribosomes from the YEp13/P1*β*-transformed D45 strain were analyzed by

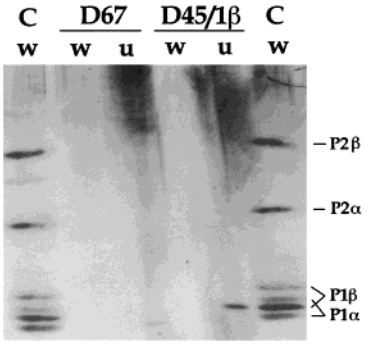

FIGURE 7: Acidic proteins in ribosomes from *S. cerevisiae* D45 and D67 strains. Ribosomes from *S. cerevisiae* D67 (D67) and D45 transformed with YEp13/P1β (D45/1β), were resolved by isoelectrofocusing. The particles were either unwashed (u) or washed in high salt buffer (w). Washed ribosomes from *S. cerevisiae* W303 were used as a control (C).

isoelectrofocusing, showing a clear band in the position corresponding to protein P1β (Figure 7). The protein is, however, removed from the particles upon washing with high salt buffers. Nevertheless, neither washed nor unwashed ribosomes from *S. cerevisiae* D67, which contains a high amount of protein P2β in the supernatant, did show any acidic protein (Figure 7).

DISCUSSION

The components of the yeast ribosomal stalk, namely the 38 kDa P0 protein and the 12 kDa acidic proteins P1α, P1β, P2α, and P2β, are usually found phosphorylated in the ribosome and dephosphorylated in the cytoplasm (*11*, *14*). The results from this report clearly show that recombinant P1α and P2β are able to bind to the ribosomes totally lacking acidic proteins and stimulate their polymerizing activity despite their nonphosphorylated state. Previous in vitro data suggested that phosphorylation was involved in the reconstitution of eukaryotic protein-deficient ribosomes by free acidic proteins (*15*, *16*, *36*). These reports, however, used unresolved mixtures of the four P proteins treated with phosphatase as well as core ribosomal particles deprived of the stalk by washing with ammonium/ethanol. It is possible that the manipulation of either the proteins or the particles could have affected the reconstitution process, thus explaining the discrepancy with the present results.

In any case, it seems that, indeed, phosphorylation is not a requirement for the in vitro interaction of the yeast acidic proteins, or at least of P1α and P2β, with the ribosome. The recombinant proteins were able to bind to the particles in similar amounts as in the wild-type ribosomes, and they remained unmodified after binding. These results agree with data from mutational studies indicating that substitution of the serine in the phosphorylation site by cysteine abolishes the capacity of the yeast acidic P proteins to be phosphorylated, while neither their competence for binding to the ribosome nor the activity of the nonphosphorylated ribosomes is affected (*17*). There are data supporting an implication of the phosphorylation of the stalk proteins in some ribosome functions (*17*, *18*, *37*). However, it seems improbable that this effect is due to either an alteration of the protein affinity for the ribosome and/or to the stability of the stalk.

Among other features, the acidic P1 and P2 proteins differ in their N-termini. While P1 proteins carry an acetylated amino end, in the P2 type the N-terminal is free (*35*). This modification is highly conserved among eukaryotic organisms and is reminiscent of the modification found in the equivalent bacterial L7/L12 proteins. This modification seems, however, not to be essential for the activity of the P1 proteins, since the inactivation of the acetylating enzyme has little effect on the ribosome activity (*35*, *38*). The results in this report confirm this conclusion, as the recombinant P1α protein, despite carrying a free N-terminus, is able to reconstitute the yeast ribosome. Moreover, the capacity of an N-formylated P2β derivative to bind to the ribosome indicates that blocking of the amino end does not have a dramatic effect on the activity of this protein either. The meaning of this highly conserved structural characteristic of the acidic proteins remains, therefore, obscure.

The results obtained using a truncated form of protein P2β^t confirm previous in vivo data obtained using protein fused to reporter genes (*39*), indicating that the most important part of the protein for binding to the ribosome is the N-terminal domain. Removal of 38 amino acids, about one-third of the protein primary structure, has a relatively small effect on its binding to the ribosome. The highly conserved C-terminal domain of the acidic P proteins is probably directly involved in their function but is not essential for ribosome interaction.

Probably, the most interesting results from this report concern the unexpected binding of protein P1α alone to acidic protein-deficient ribosomes. The analysis of ribosomes from a series of acidic protein-deficient yeast strains had previously indicated that the formation of the stalk complex seemed to require the presence of at least one protein of the each type, P1 and P2, in addition to P0 (*10*, *20*). However, the in vitro results clearly indicate that protein P1α alone is able to bind in vitro to ribosomes lacking the four acidic ribosomal proteins. Moreover, the P1α binding to deficient ribosomes is a saturable process, and it does not take place in wild-type ribosomes, indicating that it requires a suitable empty binding site in the stalk. All these data support the specificity of the in vitro P1α interaction. Nevertheless, the binding of the protein alone does not reconstitute the activity of the fully depleted particles and may even cause a slight inhibition, suggesting that the conformation of the bound protein is not fully correct.

In contrast, protein P2β is unable to bind to the ribosome unless P1α is present in the reconstitution reaction, in agreement with the in vivo results. These results suggest that there is no binding site for P2 proteins in protein P0, and they bind to the complex through the P1 proteins. This hypothesis agrees with observations of a specific interaction between P2β and P1α proteins in the absence of other ribosomal components (*31*). Alternatively, the binding of P1 could induce the appearance of a P2 site in the stalk. The characterization of a cross-link between proteins P2 and P0 in *A. salina* ribosomes seems to support the second possibility (*4*). In any case, since the activity of the particles is stimulated only when both proteins are present, P2β must induce upon binding the appropriate conformation of the whole protein complex. These results are compatible with an orderly process for the ribosomal stalk assembly, in which

the P1 proteins bind first to protein P0 and are afterward followed by the P2 proteins.

The available experimental data indicate that in normal conditions an accumulation of free P1 proteins never occurs. These proteins, and especially P1α, are very sensitive to degradation in the cytoplasm, only being stabilized by the presence of the stable P2 proteins (Nusspaumer, Remacha, and Ballesta, unpublished results). The existence of a preribosomal interaction between P1α and P2*β*, as observed in vitro, together with the structural changes that follow the formation of the complex (*31*), could explain the enhanced stability of P1α in vivo when P2 proteins are also present.

It is possible, however, to induce an accumulation of free P1*β* in the yeast D45 strain by notably increasing the gene dosage, and in these conditions the protein alone appears bound to the ribosome. In similar conditions, an accumulation of P2*β* protein in the cytosol does not result in an equivalent presence of this protein in the ribosome. The in vivo binding of P1*β* alone is weaker than the one detected in vitro, indicating that, probably, the assembly process does not take place by exactly the same mechanism than in vitro. Nevertheless, the results clearly indicate that in the cell the P1 protein is able to interact with the ribosome independently from the P2 but not the other way around.

It is quite possible that the acidic proteins are in the cell cytoplasm forming complexes among themselves as well as with other cytoplasmic polypeptides (*30*). Therefore, an independent and consecutive interaction of the P1 and P2 proteins with the ribosome is improbable. However, an assembly process brought about by the association of P1/P2 complexes approaching P0 on the P1 side would be compatible with the experimental data.

Our experimental results indicate that functionality and assembly of the different components of the ribosomal stalk are very closely correlated. The assembly and activity of the stalk is fully cooperative and needs of both types of proteins P1 and P2 to succeed: P1 proteins would provide the anchorage to the ribosome, and P2 proteins would confer functionality to the complex. This evidence supports a number of possibilities for regulation and points out the importance of this complex in eukaryotic protein translation and its control mechanisms.

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