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The amino terminal end determines the stability and assembling capacity of eukaryotic ribosomal stalk proteins P1 and P2

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ABSTRACT

The eukaryotic ribosomal proteins P1 and P2 bind to protein P0 through their N-terminal domain to form the essential ribosomal stalk. A mutational analysis points to amino acids at positions 2 and 3 as determinants for the drastic difference of *Saccharomyces cerevisiae* P1 and P2 half-life, and suggest different degradation mechanisms for each protein type. Moreover, the capacity to form P1/P2 heterodimers is drastically affected by mutations in the P2β four initial amino acids, while these mutations have no effect on P1β. Binding of P2β and, to a lesser extent, P1β to the ribosome is also seriously affected showing the high relevance of the amino acids in the first turn of the NTD α-helix 1 for the stalk assembly. The negative effect of some mutations on ribosome binding can be reversed by the presence of the second P1/P2 couple in the ribosome, indicating a stabilizing structural influence between the two heterodimers. Unexpectedly, some mutations totally abolish heterodimer formation but allow significant ribosome binding and, therefore, a previous P1 and P2 association seems not to be an absolute requirement for stalk assembly. Homology modeling of the protein complexes suggests that the mutated residues can affect the overall protein conformation.

INTRODUCTION

The stalk is a lateral protuberance of the large ribosomal subunit involved in the interaction and function of several soluble factors during translation (1). The ribosomal stalk is formed by a complex of a ribosomal RNA (rRNA)-binding protein, the prokaryotic L10 and the eukaryotic P0, and a set of acidic proteins called L7/L12 in prokaryotes and P1/P2 in eukaryotes. Protein L7/L12 binds as a homodimer to L10 forming either a pentameric L10-$(L7/L12)_2$ or a heptameric L10–$(L7/L12)_3$ complex depending on the organism (2,3). In higher eukaryotes there are two acidic protein families, P1 and P2, which interact with protein P0 as P1/P2 heterodimers to form a P0–$(P1/P2)_2$ pentamer (4,5). *Saccharomyces cerevisiae* contain two different forms in each acidic protein family, α and β, which are found in the ribosome also forming a P0-(P1α/P2β)(P1β/P2α) pentamer (6).

A notable dynamism is one of most significant features of the eukaryotic stalk. Thus, in contrast to the stability of the prokaryotic L10–L7/12 interaction (7), the eukaryotic complex is easily disassembled (8). Moreover, the ribosome-bound P1 and P2 proteins are exchanged with the free acidic proteins present in a large cytoplasmic pool (9–11) and this process, which implies changes in the P1/P2 affinity for the ribosome, is increased during protein synthesis (12). This stalk dynamism results in the presence of diverse ribosome subpopulations containing different amounts of P1/P2 proteins (13,14). The absence of P1/P2 reduces the ribosome translating efficiency to a different extent depending on the translated messenger RNAs (mRNAs), and some proteins are expressed at a higher rate by the defective ribosomes (15). Therefore, the overall cellular pattern of protein expression can be defined, among other mechanisms, by the relative proportion of each ribosome subpopulation, which is apparently determined by the metabolic activity of the cell.

To fully understand the proposed stalk-dependent regulation at the molecular level, it is indispensable to know

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the mechanism involved in the assembly and disassembly process and how it can be controlled by the cell. In this regard, there are at least two important aspects to be considered. One of them concerns the interaction dynamics between P1, P2 and P0, while the other is related to the size and control of the P1/P2 cytoplasmic pool, which must affect the equilibrium between the different ribosome subpopulations. Regarding the interaction between the stalk components, the regions determining the binding site have been characterized in both P0 (16–18) and in P1/P2 (19), but a high-resolution structure of the eukaryotic complex, necessary to fully understand the complex formation, is unfortunately missing. The crystal structure of the archaeal ribosomal stalk core structure recently reported (20) can be helpful in understanding the equivalent eukaryotic ribosomal domain, considering the significant similarity of their components. Similarly, the newly reported nuclear magnetic resonance (NMR) structure of a mammalian protein P2 homodimer (21) is an important step toward the resolution of the really biologically important P1/P2 heterodimer.

On the other hand, information available on size and control of the cytoplasmic pool of the P1 and P2 proteins in eukaryotic cells is rather scarce. There are data pointing to an effect at the transcriptional level (22). Moreover, there is a notable difference in the stability of the two acidic protein types, which may have a role in determining their relative proportion in the cytoplasm (23). Thus, while the half-life of P2 proteins is longer than 5 h, that of the P1 proteins is shorter than 15 min; nevertheless, the association of both protein types forming a P1/P2 couple protects P1 from degradation (23,24). This association is therefore essential for the existence of a pool of P1 proteins, and it is also an important fact in understanding the stalk assembly.

We previously reported that the different degradation sensitivity of both acidic protein types, P1 and P2, is determined by the first five amino acids of the protein sequence but not by the N-terminal acetylation (23). In this report, we have analyzed in more detail the degradation determinants at the N-terminal of *S. cerevisiae* P2β and P1β proteins by replacing each one of the first four residues in protein P2β by those present in the same position in protein P1β and *vice versa*. At the same time, we have checked the effects of mutations in this region on P1–P2 heterodimer formation as well as in the binding of the proteins to the ribosome. Our results, in addition to identifying more precisely the degradation determinants, have shown that modifications at the amino end of the proteins seriously affect their capacity to form P1/P2 couples as well as the protein affinity for P0.

MATERIALS AND METHODS

Strains and growth conditions

Saccharomyces cerevisiae D5 (MATα; *leu2-3, trp1-1, ura3-1, ade2-1, his3-11,15, can 1-100, RPP2β::HIS3*) and D56 (MATα; *leu2-3, trp1-1, ura3-1, ade2-1, his3-11,15, can 1-100, 3 RPP1β::TRP1, RPP2β::HIS*) were derived from *S. cerevisiae* W3031b as previously described (24,25). *Saccharomyces cerevisiae* MaV203 (MATa, leu2-3,112, trp1-901, his3Δ200, ade2-101, gal4Δ, gal80Δ, SPAL10::URA3, GAL1::lacZ, $HIS3_{UAS\ GAL1}$::HIS3@LYS2, $can1^R$, $cyh2^R$) (Invitrogen) was used as reporter host strain in the two-hybrid assay. Yeasts were grown in either rich YEP or minimal SC media supplemented with 2% glucose.

Escherichia coli XL1-Blue was used for plasmid manipulations and was grown in LB medium.

Plasmids

Constructs carrying proteins P1β and P2β mutations. The plasmids carrying protein P1βF2 and P1β5ntP2β have been previously described in refs. (26 and 23), respectively. The remaining constructs were obtained following a similar strategy (23) detailed in the Supplementary Data using oligonucleotides in Table S1.

Two-hybrid constructs. Polymerase chain reaction (PCR) DNA fragments were obtained from the cloned *RPP2B* and *PRP1B* and inserted into plasmids pBDC(*TRP1*) or pADC(*LEU2*) as previously reported (27) and is detailed in the Supplementary Data.

Genetic manipulations and recombinant DNA techniques were carried out following standard protocols (28). PCR reactions were performed with Ex Taq^{RT} polymerase (TaKaRa) and custom-made oligonucleotides (Isogen) according to re. 29.

Cell fractionation and ribosome preparation

Cell extracts and washed ribosomes from *S. cerevisiae* cells were prepared as previously described (19)

Protein analysis

Proteins were analyzed either by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or by isoelectrofocusing in 5% polyacrylamide gels using a 2.0–5.0 pH range (30) and detected either by western blotting using a specific anti-P2β or anti-P1β monoclonal antibodies (31) or by silver staining.

Protein N-terminal sequences have been obtained by Edman degradation at the Servicio de Proteómica in the Centro de Investigaciones Biológicas, Madrid.

Analysis of protein interactions in a two-hybrid assay

Two-hybrid yeast interaction assays were performed in the *S. cerevisiae* Mav203 strain, which carries the *URA3* gene under control of the GAL4 promoter (32). Pairs of plasmids expressing the activation and binding domain from GAL4 fused to the carboxyl end of the proteins to be tested (27) were co-transformed into the yeast cells and the transformants were selected on solid synthetic minimal medium free of tryptophan and leucine (SC-Trp-Leu). Protein interactions were analyzed in SC-Ura solid medium by plating dilutions of each transformed strain.

Affinity chromatography

Cell supernatant (S100 fraction) and Ni^{2+}–NTA resin (Invitrogen) were mixed in a ratio of 1 mg of extract per 35 µl of resin, previously equilibrated in LY buffer (10 mM Tris–HCl, pH 7.5, 20 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$). The sample was incubated for 1 h. at 4°C with continuous shaking and then the mixture was loaded in a column (10 cm × 1 cm), and the flow through fraction was collected. The column was subsequently washed first with 10 vol of buffer LY, then with 10 vol of 10 mM imidazol in buffer LY. Finally, the resin-bound proteins were eluted with 4 vol of 100 mM imidazol in the same buffer and the eluted fraction was collected.

Protein complex structure modeling

Models of *S. cerevisiae* P1/P2 heterodimers and the stalk pentamer have been generated by MODELLER 9.8 (33) using human NTD-P2 (2W1O) (21), and archaeal stalk complex (3A1Y) (20) as templates.

Molecular dynamics simulations were run in NAMD (34) for complexes fully solvated with explicit water molecules, during 20 ns at 298 K under constant pressure and periodic boundary conditions. Further details are available as Supplementary Data.

RESULTS

Residues at positions 2 and 3 in the amino acid sequence are major determinants for degradation sensitivity in proteins P1β and P2β

One significant difference compared to prokaryotes in the structure of the NH_2-terminal end of eukaryotic acidic P1 and P2 proteins is acetylation, which takes place at Ser 2 after removal of the starting methionine only in the P1 proteins (35). Amino-terminal acetylation has been considered as a degradation signal in some proteins (36,37), and, therefore, the effect of modifications in Ser 2 and neighboring residues on the sensitivity to degradation in protein P1β was explored. Proteins carrying a mutation in position 2, P1βF2, and a double mutation in positions 2 and 4, P1βK2L4, as well as a P1β derivative with the five first amino acids substituted by the equivalent sequence from P2β, P1β5ntP2β, were constructed (Table 1). The mutated genes were expressed in *S. cerevisiae* D456 which lacks the wild-type P1β and the two P2 proteins (15). In this strain, the expressed mutated P1β proteins do not have their P2 partner and then are unable to bind to the ribosome. They rest unprotected in the cytoplasm and accessible to cellular proteolytic activities (23). As shown in Figure 1A, a western blot analysis of transformed D456 total cell extracts showed that mutation of S2F resulted in an increase of the amount of P1β to the parental W303 level while the wild-type protein was fully degraded in the same conditions. Additional mutations (P1βK2L4 and P1β5ntP2β) did not cause a further P1β increase in the cell extracts (Figure 1A).

Considering the previous results. it could be expected that introducing a Ser at position 2 in P2 proteins destabilizes these highly stable proteins. However, preliminary experiments showed that a Ser at position 2 or/and position 4 in protein P2β had only a minor effect on the protein stability (data not shown, see below). Therefore, a more extensive analysis was carried out basically by replacing each one of the first four residues in protein P2β by those present in the same position in protein P1β (Table 1). The mutant proteins were expressed in *S. cerevisiae* D567, which lacks P2β and both P1 proteins (15). In this strain, the expressed P2β protein lacks the corresponding P1 partner and remains free in the cytoplasm. Estimation of the protein in the extracts confirmed that the amount of P2β is practically unaffected by the introduction of a Ser at position 2 (protein P2βS2) and is somewhat decreased by an additional Ser at position 4 (protein P2βS2S4) (Figure 1A). In contrast, the protein is absent in the extracts from all mutants carrying an Asp in position 3 (P2βD3, P2βS2D3, P2βD3S4 and P2βS2D3S4).

Table 1. Mutations used in this work

Protein	Mutation	N-terminal sequence		
		Encoded[a]	Expected[b]	Sequenced[c]
P1β	None	M**S**DSI	Ac-**S**DSI	Blocked
P1βF2	S2F	M**F**DSI	M**F**DSI	M**F**DS
P1βK2L4	S2K,S4L	M**K**D**L**I	M**K**D**L**I	N/D
P1β5ntP2β	S2K,D3Y,S4L,I4A	M**KYLA**	M**KYLA**	N/D
P2β	None	MKYLA	MKYLA	MKYL
P2βS2[d]	K2S	M**S**YLA	Ac-**S**YLA	M**S**YL, SYLA, Blocked
P2βS2S4	K2S,L4S	M**S**Y**S**A	Ac-**S**Y**S**A	Blocked
P2βS2D3S4	K2S,Y3D,L4S	M**SDS**A	Ac-**SDS**A	N/D
P2βS2D3	K2S,Y3D	M**SD**LA	Ac-**SD**LA	N/D
P2βD3	Y3D	MK**D**LA	MK**D**LA	MK**D**L
P2βD3S4	Y3D,L4S	MK**DS**A	Ac-K**DS**A	N/D

[a]Sequence derived from gene nucleotide sequence. Mutated positions are in bold.
[b]Sequence expected from N-terminal processing rules.
[c]Sequence determined by Edman degradation in proteins able to bind to ribosomes (Figure 2).
[d]Sequencing has shown the presence of an N-terminal blocked form in minor amounts while two unblocked forms (M**S**YL, SYLA) are present in similar amounts (Figure 2).

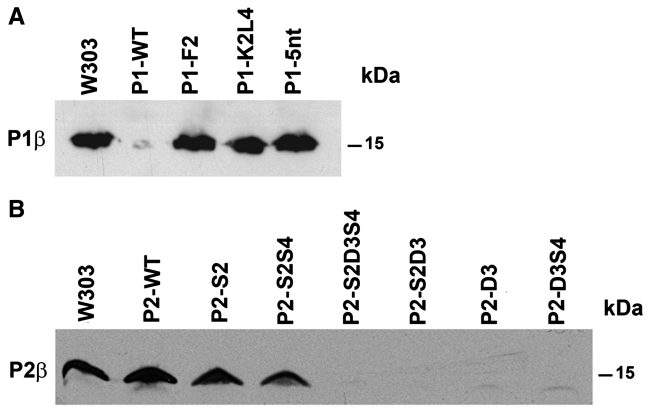

Figure 1. Estimation of soluble protein P1β and P2β mutants in *S. cerevisiae*. Total extracts from *S. cerevisiae* D456 (**A**) or D567 (**B**) transformed with plasmid expressing the indicated proteins were resolved by SDS–PAGE and the proteins detected by immunoblotting using a specific monoclonal antibody to either protein. Extracts from the parental W303 strain were used as control.

Effect of N-terminal mutations on protein binding to the ribosome

The P1β and P2β mutant series were expressed in *S. cerevisiae* D56, which lacks proteins P1β and P2β but contains their respective protein partner, P2α and P1α (24), and the ribosome bound stalk components were analyzed by isoelectrofocusing (30). The S2F and S2K,S4L mutations in P1βF2 and P1βK2L4 had no detectable effect on the amount of ribosome-bound protein, and it had previously been found that a single S4R mutation did not affect binding to the ribosome either (38). In contrast, protein P1β5ntP2β was not detected in the particles (Figure 2A). In the P2β series, introduction of a Ser at position 2 resulted in a duplication of the mutated protein bands, which correspond to different N-terminal processed forms of the protein (Table 1 and Supplementary Data). The K2S mutation had a limited effect on the protein ribosome-binding capacity. In contrast, the protein in the ribosome was notably affected by mutating position 3 or by a double mutation in two out of the three analyzed amino acids, and was totally abolished by changing the three of them simultaneously (Figure 2B and Supplementary Figure S1). It must be noted that the bound mutant proteins are functional since they stimulate strain D56 growth (Supplementary Data, Table S2).

The P2β mutants were also expressed in *S. cerevisiae* D5, which only lacks their parental wild-type protein. In this case, after transformation the cells will contain a full complement of stalk components and it will be possible to test whether this fact somehow affects the binding of the mutated proteins to the ribosomes. The ribosomes from both transformed strains were analyzed by SDS–PAGE and the proteins estimated by immunoblotting (Figure 2C). It was clear that the presence of a complete stalk favored the binding of proteins P2βS2S4, P2βS2D3 and P2βD3S4, which were present in higher amounts in ribosomes from strain D5. In contrast, the triple mutant protein was not found in either strain.

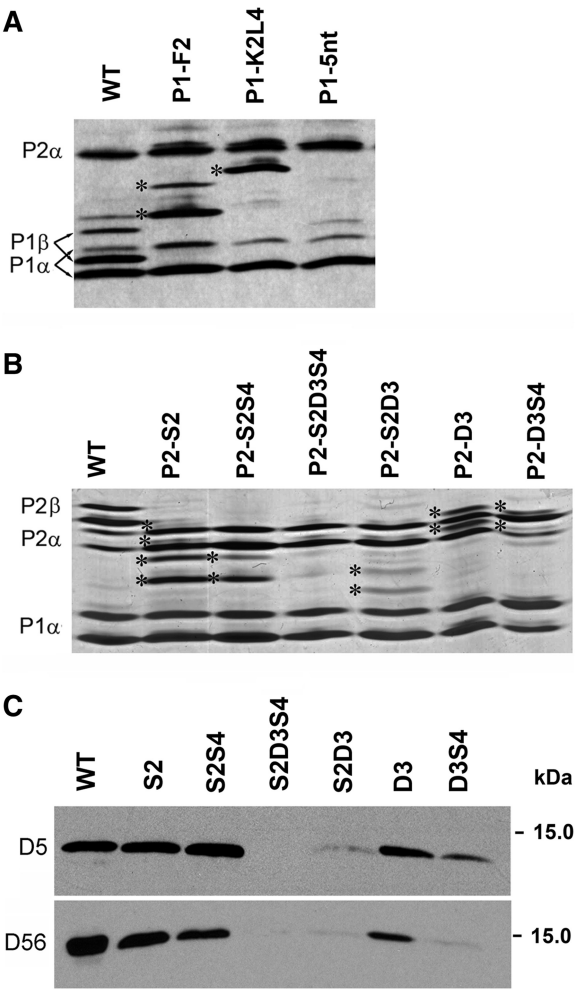

Figure 2. Detection of ribosome-bound protein P1β and P2β mutants. (**A** and **B**) Purified ribosomes from *S. cerevisiae* D56 expressing either the P1β (A) or the P2β (B) mutants were resolved by isoelectrofocusing and the proteins detected by silver staining. The P proteins usually appear as a double band, which correspond to the phosphorylated (lower band) and non-phosphorylated (upper band) forms. The mutated proteins are marked by an asterisk in the stained gel. (**C**) Ribosomes purified from strains D5 and D56 transformed with the P2β mutants were resolved by SDS–PAGE and the proteins detected by immunoblotting using a specific anti-P2β monoclonal antibody.

The formation of P1α/P2β heterodimers is affected by the N-terminal mutations

The capacity of the P1 and P2 proteins to form P1/P2 heterodimers, preferentially P1α/P2β and P1β/P2α in the case of *S. cerevisiae* (39), suggested that the formation of these associations is a requirement for binding to the ribosome. Since some of our mutant proteins are either totally or partially affected in ribosome binding we checked whether their ribosome binding defect parallels their capacity to associate with the partner P1α protein. Thus, we tested the interaction of the P1β and P2β mutant series with proteins P2α and P1α, respectively, using the two-hybrid technique. Either the tests were carried out with the mutant proteins fused to the GAL4 binding

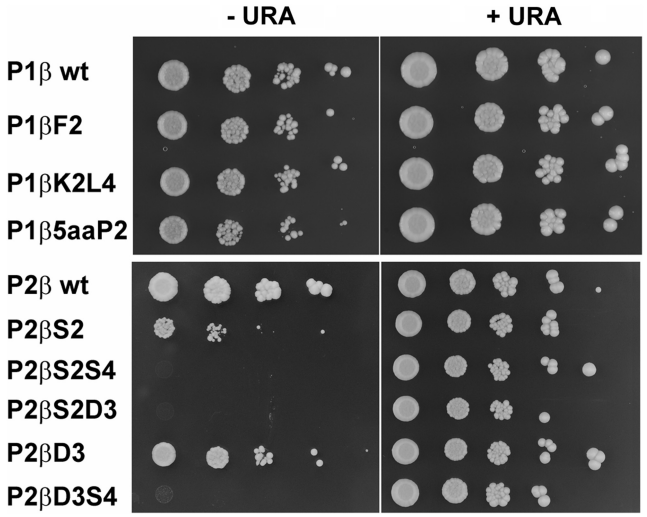

Figure 3. Interaction of P1β mutants with protein P2α (upper panel) and P2β mutants with protein P1α (lower panel) estimated by the two-hybrid test. *S. cerevisiae* MaV203 was co-transformed with the construct expressing the different mutants fused to the GAL4 binding domain and the plasmid expressing the corresponding wild-type protein fused to the GAL4 activating domain. Serial dilutions of the transformed cells were plated on SC medium lacking uracil (left panels). The extent of growth in these conditions is proportional to the interaction between the tested proteins. As a control for cell viability, a similar test was carried out on plates carrying uracil (right panels).

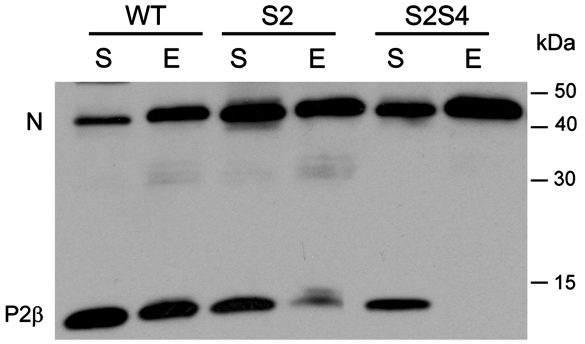

Figure 4. Estimation of the interaction of P2β mutants with protein P1α using affinity chromatography. Supernantant S100 extracts (S) from *S. cerevisiae* cells expressing simultaneously the indicated P2β proteins and P1α-His6 were adsorbed in Ni^{2+}–NTA resin. After washing, the fractions eluted with 100 mM imidazol were analyzed by SDS–PAGE and the P2β proteins detected by immunoblotting (E). A non-characterized protein detected by the antibody (N) can be used as loading control.

domain (GAL4BD) and the corresponding wild-type partner to the activation domain (GAL4AD), or vice versa, obtaining in both cases similar results.

An interaction of P2α with the P1β mutants was detected in all cases, even with P1β5ntP2β, which is unable to bind to the ribosome (Figure 3A). In the P2β series, the wild-type P1α was able to interact only with P2βS2 and P2βD3, though to a reduced extent, especially in the first case. All the other tested proteins totally failed to allow cell growth and, therefore, seemed unable to interact with P1α (Figure 3B). An immunoblotting analysis confirmed that the mutant proteins fused to the Gal4 domain were present in the *S. cerevisiae* MaV203 cell extracts (Supplementary Data and Supplementary Figure S2).

A comparison of the two-hybrid and ribosome binding tests (Figures 2 and 3) indicated that there was not always a direct relationship between the capacity for ribosome binding and for P1/P2 association. Thus, protein P1β5ntP2β, which showed a practically normal level of association with P2α, was totally absent from the ribosome. In contrast, protein P2βS2S4, present in significant amounts in ribosomes from D56 and practically at the control level in D5, did not show interaction with P1α in the two-hybrid system. Since the P2β mutant result was totally unexpected, it was confirmed using affinity chromatography as an alternative experimental approach for testing interactions. C-terminal His6-tagged P1α was expressed in *S. cerevisiae* D5 together with P2βS2, P2βS2S4 or wild-type P2β. The supernatants (S100 fractions) from the cells were loaded in a Ni^{2+}–NTA column, and the amount of each P2β specifically bound to resin was estimated by immunoblotting. As shown in Figure 4, while wild-type P2β is strongly retained by P1α-His6 in the column as expected, mutant P2βS2 is drastically reduced and mutant P2βS2S4 totally absent in the eluted fractions.

Modeling *S. cerevisiae* P1–P2 heterodimers and the (P0,P1α-P2β,P1β-P2α) pentamer

An accurate interpretation of the previous results would be notably helped by a high-resolution structure of the yeast stalk, which is not yet available. However, the recently reported structures of the human P2 homodimer (21), and the archaeal pentameric $[P0(P1)_2(P1)_2(P1)_2]$ stalk complex (20), have allowed homology modeling of the structure of the equivalent yeast protein complexes. As expected, the two yeast heterodimer structures, P1α-P2β P1β-P2α, are quite similar (Supplementary Figure S4A). They slightly diverge at the carboxyl terminal domains but the α-helix 1 nicely overlaps in both models. Since the P1 and P2 NTD sequence differ notably, the structure of the yeast heterodimers lacks the symmetry of the human P2 homodimer. Thus, the position of the P1 and P2 N-terminal in relation to the whole structure is significantly different (Supplementary Data and Supplementary Figure S4B).

The yeast stalk structure (Supplementary Figure S5) has been modeled taking into consideration two facts: (i) first, the P1α/P2β and P1β/P2α heterodimers bind to the first and second α-helix in the P0 C-terminal spine, respectively (17) and (ii) the data from the human stalk support that the P1 proteins from both heterodimers are probably facing each other in the pentamer (21). The modeled *S. cerevisiae* pentamer shows differences compared to the equivalent human structure mainly due to the disparity of the two yeast heterodimers. Among others, the angle formed by the two P0 α-helices is considerably more acute in yeast pentamer (Supplementary Data and Supplementary Figure S6).

DISCUSSION

Structural stability of the individual proteins

It was previously shown that the radical difference in protease sensitivity of P1 and P2 proteins lays in the first five amino acids of each protein (23). This report shows that just the mutation S2F totally abolishes the high protease sensitivity of protein P1β. As previously reported (35,40), the presence in P1β of a serine in the second position of the amino acid sequence induces removal of the starting Met and acetylation of the exposed NH2-end. In contrast, the presence of a bulky Phe residue after the initiator Met blocked the N-amino end processing of proteins (Table 1). We have previously shown that N-terminal acetylation is not a degradation determinant for P proteins (23); therefore, cleavage of the first Met seems to be a requirement for degradation of P1β, and probably for P1 proteins in general since all of them carry a small uncharged amino acid in the second position (41,42), which must induce N-amino end processing (43,44).

On the other hand, replacing the Lys2 in P2β, an N-amino end processing blocker, by a serine has a limited effect on protein stability, in spite of the fact that the mutation induces extensive processing of the protein N-terminal. In contrast, mutation in position 3, which did not cause N-terminal processing (Table 1), results in a drastic decrease of protein P2β stability either in the presence or in the absence of a serine in the second position.

These results indicate that the actual degradation determinants are different in both protein types. Thus, amino acids in positions 2 and 3 are determining the half-life of proteins P1β and P2β, respectively. For this reason, the same four initial amino acids, MKDL, which contains a P1β stabilizing Lys in position 2 and a P2β destabilizing Glu at position 3, can have an opposite effect in the two proteins, inducing stability of P1βK2L4 and instability in P2βD3. Probably, other elements may exist in the proteins, perhaps secondary or tertiary structural features, which together with the N-terminal determinants, define their different degradation sensitivity. Alternatively, the existence of different degradation mechanisms for each protein type cannot be excluded.

P1–P2 heterodimer formation

The most interesting observation regarding the capacity of the 12 kDa acidic stalk proteins to form P1–P2 heterodimers is the sensitivity of the process to mutations at the amino end as well as the different response of the two protein types to the modifications. While most P2β mutants are unable to associate with their partner protein, similar modifications do not affect the P1 association capacity.

The association of two human P2 proteins to form a homodimer takes place mainly through symmetrical hydrophobic interactions between α-helices 1, 2 and 3 from both components (21). The modeled yeast heterodimer structures are more asymmetric and the N-ends of P1 and P2 proteins have a clearly different structural environment. The P2 α-helix 1 is shorter than in P1, and its N- terminal is in a more internal position that provides more stabilizing interaction possibilities for the first amino acids (Supplementary Figure S4B) and might explain the different effects of similar mutations on both proteins. Thus, while in P1β Ser2, which is actually the first residue due to the removal of the initiating methionine, and Ser4, can hardly be involved in important interactions, Met1 and Lys2 in P2β can directly interact through hydrogen bonding with Asp15 and Leu11 lateral chains in P1α α-helix 3 (Figure 5A). Residues Tyr3 or Leu4, whose mutation is required for total elimination of the P2β associating capacity, are, however, oriented toward the inside and they cannot be directly involved in the interaction with P1α but their substitution probably changes the protein conformation. Thus, Leu4 is part of the hydrophobic core formed by P2β helices 1, 3 and 4, which can be seriously disturbed in the mutants. Taking into consideration all these facts, it is likely that the mutations in the P2β N-end, in addition to directly affecting the interaction with P1α, seem to alter the first turn of helix 1 inducing a conformational change that apparently affects the stability of the whole complex. The structural similarity of both yeast heterodimers as well as high sequence homology of the N-terminal domains of the proteins from both families make it highly probable that data obtained from P1β and P2β can be extended to P1α and P2α, respectively. In any case, confirmation of our structural predictions will require the crystal structure of the yeast stalk complex, which we hope will not take too long to be resolved.

As a whole, the data show the great structural relevance of the first turn of α-helix 1 in P2 proteins, but not in P1 proteins for heterodimer stability, and stress the differences in the structural role of the two proteins families have previously been reported using other experimental approaches (45).

Assembly into the yeast ribosomal stalk pentamer

In the modeled pentamer the main structural elements participating in the interaction with P0 are α-helices 1 and 4 from the N-terminal domain of the four acidic proteins (Supplementary Figure S5). Our results show that individually the first four amino acids of P2β, which practically form the α-helix 1 first turn, partially contribute to the stability of the complex and all together are essential in maintaining the association of the heterodimer with stalk. In fact, the four amino acids show potential interactions with residues in the P0 α-helix as well as in P1α-helix 1 and P2β α-helix 3 (Figure 5B).

In contrast, modification of positions 2 and 4 in P1β does not affect protein binding to the ribosome and apparently does not contribute much to the stability of the complex. Actually, in the modeled pentamer structure the first three residues of the protein are apparently not included in α-helix 1 (Supplementary Figure S5). A more extensive modification including the first five residues, which in this case affects helix 1 (Figure 5C), abolishes the protein capacity to bind to the ribosome. It seems, therefore, that as in the heterodimer formation, the

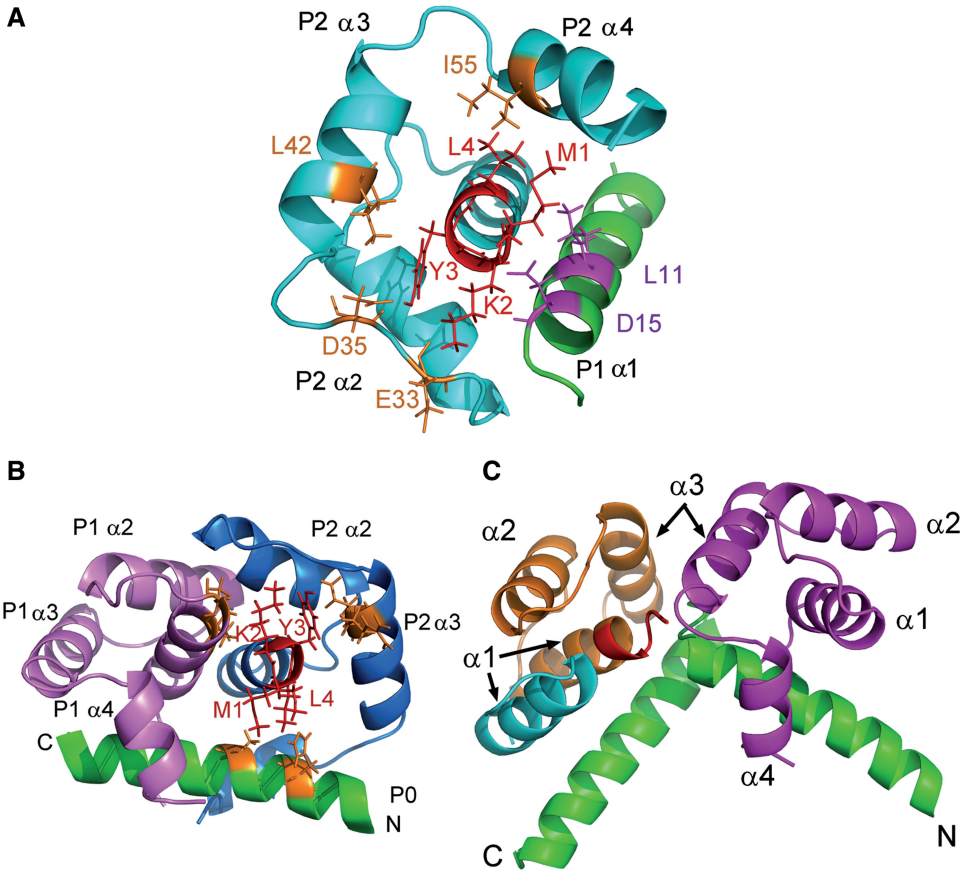

Figure 5. Close view of the amino ends from protein P2β (**A** and **B**) and P1β (A and **C**) in the modeled *S. cerevisiae* stalk heterodimer (A) and pentamer (B and C) shown in Supplementary Figures S4 and S5. (A) Protein P2β α-helices 1, 2, 3 and 4 (cyan) and P1α α-helix 3 (green) are shown. Lateral chains, names and sequence position of the first four amino acids from P2β are shown (red) as well as those from the nearer amino acids in other P2β regions (orange) and in P1α α-helix 3 (magenta). (B) Helices from protein P2β (blue), P1α (magenta) and P0 (green) surrounding the first amino acids from P2β α-helix 1 (red) are shown. The closest residues to these amino acids in the surrounding helices are marked in orange. (C) A closer view of the first amino acids (red) from protein P1β (orange) is shown. The closer structural elements from proteins P2α (cyan), P1α (magenta) and P0 (green) are shown. The amino and carboxyl end of the P0 fragment are marked in panels. Note that the orientation of the model is opposite to that shown in Supplementary Figure S5.

initial amino acids of P1 and P2 proteins are not equally relevant.

The differences found in ribosome binding of P2β mutants in *S. cerevisiae* D5 and D56 (Figure 2C) indicate that the presence of the protein P1β, absent in the D56 cells, facilitates the association of the defective proteins to the ribosome. Protein P1β can form P1β/P2α heterodimers that allow the assembly of a complete (P1α/P2β)–P0–(P1β/P2α) pentamer, which is missing in strain D56 and seems to stabilize the binding of the defective mutant P2β–P1α couples. These results support that the two heterodimers forming the ribosomal stalk are not independent as has been proposed for the human ribosomes (21).

Role of heterodimers in pentamer assembly

As commented on previously, P1 and P2 are able to form heterodimers in solution, and it is assumed that their interaction with the ribosome takes place in the associated state. If this is so, there must be a direct relationship between the protein capacity to form heterodimers and to bind to the ribosome. In general, our results are in agreement with this statement but not in all cases. Some P2β mutants are detected as bound to the ribosome in high amounts, but are seriously or even totally affected in their capacity to interact in solution with P1α. Mutant P2βS2S4 is probably the clearest example. This protein was unable to interact with P1α and P2α or even to form homo-dimers (Figure 3 and Supplementary Figure S3 in Supplementary Data) but was found in high proportions in the washed D56 and D5 ribosomes. Also, mutant P2βS2 was present in the ribosomes of D5 and D56 in normal amounts but showed an important reduction in its capacity to interact directly with P1α. It seems, therefore, that although wild-type P1 and P2 proteins are usually associated as highly stable P1/P2 heterodimers and interact with P0 as couples, a strong association is not an essential requirement for forming a functional stalk in the ribosome. The existence of weak associations, not detectable by standard techniques, which is stabilized upon binding to P0, cannot be excluded. Actually, the association of the acidic proteins with the ribosome, like the binding of P0 (46–48), might require the help of assembly factors, which can facilitate the process in the

case of acidic proteins which do not form stable couples in solution.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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