Supplementary data to

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

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MATERIALS AND METHODS

Plasmids

Constructs carrying proteins P1β and P2β mutations

First, a DNA fragment (ORF fragment) from the RPP1B and RPP2B genes containing a large stretch from the 3’UTR and the complete ORF starting at the initiator ATG from both genes, was obtained by PCR using mutagenic oligonucleotides (Table S1) at the NTD encoding region and a universal primer at the 3’ distal end. In some cases, an NruI restriction site was included upstream of the ATG (Table S1). Secondly, a DNA fragment (5’UTR fragment) was similarly obtained starting from the nucleotide just ahead of the initiator ATG and comprising more than 450 bp upstream of the 5’UTR region using the 5’UTR1B and 5’UTRP2B oligonucleotides, respectively (Table S1) at the ATG proximal end and a universal primer at the distal end. This fragment was common for all the constructs of the same protein. Both fragments were obtained using the cloned corresponding genes as a template (1). Both fragments were digested with appropriate restriction enzymes, HindIII and eventually NruI for the ORF fragment and PstI for the 5’UTR fragment, to yield sticking ends at the distal extremes, which would be joined to the vector, and blunt ends at the proximal terminals, which would link the 5’UTR to the initiator ATG. The resulting restricted fragments, a 638 bp long ORF fragment and a 350 bp 5’UTR fragment in the case of P2β mutants, were simultaneously subcloned in a unique ligation reaction into the 4.6 Kb PstI-HindIII fragment from vector pFLKan. The pFLKan vector was derived from plasmid pFL38 (2) by removing the URA3 marker and inserting the KanR gene in the same place. In the case of the P1β mutant, a 900 bp ORF fragment and a 1030 5’UTR fragments were digested with BamHI and HindIII, respectively, and subcloned in plasmid pFL36 (LEU2) (2).

Two-Hybrid Constructs. PCR DNA fragments were obtained from the cloned RPP2B and PRP1B using the same mutagenic oligonucleotides described in the previous section as primer for the initiator ATG region and oligonucleotide NewRev8 (Table 2) for the CTD region. This oligo also carried an NruI site, which upon restriction removes the region encoding, the last 21 amino acids, from the P2β sequence. The PCR fragments were cloned into the NruI site of either pBDC(TRP1) or pADC(LEU2), which encode the GAL4 binding and activation domain respectively (3). The mutant constructs were tested for interaction with the native RPP1A, RPP2B and RPP0 genes equally fused to the same vectors previously obtained (4)). In all the constructs the Gal4
domains are fused to the CTD of the proteins in order to leave their NTD free for interaction. It should be noted that the last 22 aa of both acidic proteins were absent in all the constructs since this strongly acidic region was previously found to induce high non-specific background in the interaction assays (5,6).

| Table S1 |
| Oligonucleotides used for proteins P1β and P2β mutation and two-hybrid constructs |
| OLIGO | SEQUENCE | MUTATION |
| Protein P1β |
| 1BK2L4 | ATGAAAGACTTAAATTATTTCTTGCTGCT | S2KS4L |
| 5'UTR1B | TTTCTTCTTATGTGTGTGTATTTCT | |
| Protein P2β |
| 2BS2 | TC7CGCA4TGCTTACTTACTGCTGCTTTACTTAA | K2S |
| 2BS2S4 | TC7CGCA4TGCTTACTTACTGCTGCTTTACTATTTG | K2S,L4S |
| 2BS2D3S4 | TC7CGCA4TGCTGACTCCTGCTGCTTTACTTATTG | K2S,Y3D,L4S |
| 2BD3 | TC7CGCA4TGAAAGACTTACTGCTGCTTTACTTATTG | Y3D |
| 2BS2D3 | TC7CGCA4TGCTGACTCCTGCTGCTTTACTTATTG | K2S,Y3D |
| 2BD3S4 | TC7CGCA4TGAAAGACTTACTGCTGCTTTACTTATTG | Y3D,L4S |
| 5'UTR2B | TTCTGTTTGTGATTAATAGATA | |
| NewRev8 | AATCGCA4TATTCAGCATACGACAGCCACCCGCGC | |

In Bold, mutagenic nucleotides. In italics, the NruI restriction site

**Protein complex structure modelling**

Free *S. cerevisiae* P1/P2 heterodimers were modelled with program Modeller 9.8 (7) using the human P2 (PDB 2W1O) (8) as a template plus additional information from the archaeal pentamer (PDB 3A1Y chain A) (9) for the P1 protein. The [P0,P1A-P2B,P1B-P2A] pentamer was modelled using the archaeal structure (PDB 3A1Y) (9) as template.

**Molecular dynamics.**

Hydrogen atoms in standard protonation state were added to modelled structures with the Molprobity (10) web server, which also used this for minimizing the number of atom overlapping due to Asn, Gln, or His residue flips.

Protonation states of ionizable groups at pH 6.8 were calculated using the H++ (11,12) server. System topology set-up input files were generated with the CHARMM-gui server and standard atomic charges and radii for all the atoms were assigned according to the CHARMM force field (version 27) (13,14) using the program CHARMM version34b1 (15). The dimers were fully solvated to a box size of 61 Å × 64 Å × 55 Å, and the pentamer to a box size of 67 Å × 139 Å × 66 Å with the program VMD (16) using TIP3P (17) water molecules. Counter ions were also added to maintain electro neutrality plus enough extra Na⁺ and Cl⁻ to give a 0.5M solution. Water molecules were relaxed for 1000 steps, followed by an equilibration MD of 200ps applying harmonic restraints to the protein atoms and rising the temperature up to 298K allowing volume fluctuations.

**Simulation details.** MD simulation was run in NAMD (18) with a non-bonded interactions calculation cut-off of 12 Å. The production run (20ns) was conducted at
298K using a Langevin temperature piston (19) at constant pressure. Particle mesh Ewald (20) electrostatics settings were applied along the SHAKE algorithm (21) with a 2-fs time step.

RESULTS AND DISCUSSION

Amino end processing of the mutant proteins

In the P2β mutant series, it must be noted that in the case of protein P2βS2, four instead of two bands were found in the isoelectrofocusing of the ribosomal stalk proteins (Fig 2B and Fig S1). Usually, the presence of a serine in position 2 in a yeast ribosomal protein amino acid sequence facilitates the removal of the initiating methionine and acetylation of the Ser residue (22) as has been shown for yeast P1 proteins (23). Edman degradation sequencing has shown that the two upper bands are N-terminal blocked proteins, which must correspond to the expected phosphorylated and non-phosphorylated forms of a fully processed P2β with an acetylated serine as the starting residue. The lower bands contain a mixture in similar proportion to the unprocessed mutant protein (MSYL) and the processed protein carrying an unblocked serine as first amino acid (SYLA) (Table 1). It seems that in P2β, the K2S mutation, which reduces the protein pI (lower bands), induces a partial elimination of the first Met. In addition, a fraction of the processed form is apparently acetylated at the serine amino terminal increasing the protein pI (upper bands). As expected, mutation of serine 2 in P1β suppresses N-terminal processing and the mutated protein carries the first Met residue (Table 1).

These results show that the N-terminal modification of P2β induced by the presence of a serine in position 2 is less efficient than in P1 proteins and suggest that additional structural elements affect this process. This conclusion is confirmed by the data from the P2βS2S4 protein sequencing. In this protein, the presence of a second serine in position 4 generated only two blocked bands, which yielded no sequence by Edman degradation sequencing. It seems that in this double mutant protein the processing is faster and more efficient than in P2βS2.

Mutant proteins are functional

To test whether ribosome bound mutant P2β proteins are functional, the growth rate of transformed S. cerevisiae D56 cells individually expressing P2βS2, P2βS2S4, and P2βD3, was estimated in glucose minimal liquid medium. Cells that express either native P2βwt or the obviously inactive P2βS2D3S4 were used as positive and negative controls. The doubling time relative to the parental W303 parental strain is shown in Table S2. The three proteins stimulate D56 cell growth to an extent roughly proportional to the amount of protein found in the ribosomes. Protein P2βS2 is as functional as P2βwt, indicating that different forms of the mutant protein found in the ribosome, as commented on previously, are equally active.
The mutated P2β proteins are present in the two-hybrid tests

To exclude the failure to detect an interaction with some P2β mutants could be due to a differential degradation sensitivity (Fig 1), total extracts of the transformed S. cerevisiae MaV203 cells were resolved by SDS-PAGE and the proteins were detected by immunobloting using a specific antibody to P2β. A band moving to the position expected for the fused P2β-Gal4BD was detected in similar amounts in all samples (Fig S2).

Protein P2βS2S4 neither interacts with P2α nor forms homodimers.

The presence of P2βS2S4 in the ribosomes, in spite of being unable to interact with protein P1α, raised the question of whether this mutant protein could either form heterodimers with P2α, also present in strain D56, or self-dimerize forming protein couples that could interact with the ribosomal particle. Although the available experimental data do not support these possibilities, we have tested them in a two-hybrid assay. As expected, protein P2βS2S4 did not show an indication of either self-dimerization or association with P2α (Fig S3).

Modelling of S. cerevisiae P1-P2 heterodimers and the [P0,P1α-P2β,P1β-P2α] pentamer.

As described in the main text, among the different possible orientations of the two P1-P2 heterodimers, P1α-P2β and P1β-P2α, the yeast stalk pentamer was modelled providing that the P1 NTD α-helices 3 from both P1-P2 heterodimers were interacting in the complex. Lee et al have recently reported experimental data indicating that this α-helix contains a number of conserved hydrophobic residues which establish an interaction between the P1 proteins from the two heterodimers in the human stalk pentamer (8). In the yeast pentamer model a similar interaction is also possible since Ile 39 and Ile 43 from P1α and Val 38 and Val 42 from P1β are in the appropriate location of the respective α-helices 3 (Fig S4). Nevertheless, there are significant differences between the human and yeast models. Thus, the relative position of the two heterodimers, especially α-helices 3 and 4, is clearly different. The distance between α-helix 3 in P1α and P1 β is larger and the angle of the turn formed by the two α-helices

| Strain               | Relative doubling time |
|----------------------|------------------------|
| W303                 | 1 (86 min)             |
| D56+ P2βwt           | 1.3                    |
| D56+ P2βS2           | 1.3                    |
| D56+ P2βD3           | 1.4                    |
| D56+ P2βS2S4         | 1.7                    |
| D56+ P2βS2D3S4       | 2.0                    |
from the P0 fragment participating in the pentamer is sharper than in the human model (S5). These changes tend to bring closer the four acidic protein CTD’s (not included in the model) and probably confine them into a smaller space. This effect is also favoured by the position of α-helix 4 from P2β and P1β as compared with the helices in the human model (Fig S5). In spite of the high flexibility of the hinge region joining the NTD and CTD in the P proteins, this situation seems to reduce the range of action of the proteins. Since apparently a P protein CTD function is to look for soluble factors facilitating their ribosome binding, this situation apparently works against yeast stalk efficiency. However, our present understanding of the way the stalk is working is still rather limited and additional information is required to fully understand the meaning of the structural differences among organisms, like the existence of different forms of P1 and P2 proteins in some eukaryotes like *S. cerevisiae*.

![Western blot](image1.png)

Fig S1. Western blot of isoelectrofocusing gels of ribosomes from strain D56 expressing the indicated P2β mutants resolved as described in Fig 2. Proteins were detected using a monoclonal antibody specific to protein P2β.

![Total extracts](image2.png)

Fig S2.- Total extracts from *S. cerevisiae* MaV203 cells used for the two-hybrid assay shown in Fig 4 were resolved by SDS-PAGE and the presence of the mutant P2β proteins fused to the GAL4BD was detected using the anti-P2β monoclonal. A band in the expected position around 32 kDa was detected in all samples.
Fig S3.- Interaction of P2β mutant proteins with themselves and with protein P2α was estimated by the two-hybrid test as described in Fig 3. *S. cerevisiae* MaV203 was co-transformed with the construct expressing the indicates mutant proteins fused to the GAL4 binding domain and the plasmid expressing either the same mutant protein (Panel A) or wild-type protein P2α (Panel B) fused to the GAL4 activating domain. Serial dilutions of the transformed cells were plated on SC medium lacking uracil (Left panels). Interactions of P1α with P2β and of P2α with P1β were used as positive controls in Panels A and B, respectively. Similarly, self-association of wild-type P2β and the P2α/P2β association were the corresponding negative controls. The extent of growth in these conditions is proportional to the interaction between the tested proteins. As a control for cell viability, the same strains were grown on plates carrying uracil (Right Panels).

Fig S4.- A.- Overlapping of the predicted structures of the *S. cerevisiae* P1α (orange)-P2β (yellow) and P1β (cyan)-P2α (blue) heterodimers. The position of the four α-helices in each protein as well as the Amino end (N) is indicated. B.- Different views of the P1α-P2β heterodimer showing the different environment of the respective N-ends including the first four amino acids (red).
Fig S5.- Colour coded modelled structure of the *S. cerevisiae* stalks pentamer. Protein P0 is in green, P2β in light blue, P1α in pale yellow, P1β in orange and P2α in cyan. In the modelled structure the α-helix 3 of P1 proteins from the two heterodimers are facing each other and the amino acids possibly involved in the interaction are indicated. The contact regions between P1/P2 and P0 are marked in blue, and those between the P1 α-helices 3 are in magenta. The conserved YP residues (red) joining the two α-helices in the P0 protein fragment (green) are shown. The amino (N) and carboxyl (C) ends of the P0 fragment are also marked.

Fig S6.- Differences between *S. cerevisiae* and *H. sapiens* ribosomal stalk pentamer models. The figure shows the region corresponding to protein P0 and the interacting α-helices 4 from the P1 and P2 proteins in the modelled stalk pentamer from *S. cerevisiae* (A) and from *H. sapiens* (B). The human structure has been derived from Fig S4A in a recent report on *H. sapiens* stalk (8). An equivalent view of both structures is shown. An estimation of the angle formed by the two P0 α-helices as well as the orientation of the clearly visible α-helices 4 is shown.
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