The ribosomal P-proteins of the medfly <i>Ceratitis capitata</i> form a heterogeneous stalk structure interacting with the endogenous P-proteins, in conditional P0-null strains of the yeast <i>Saccharomyces cerevisiae</i>

Mary Elizabeth Gagou, M. A. Rodriguez Gabriel, Juan P. G. Ballesta and Sophia Kouyanou*

University of Athens, Department of Biology, Division of Genetics and Biotechnology, Panepistimiopolis, Kouponia, 15701 Athens, Greece and Centro de Biologia Molecular Severo Ochoa, CSIC and UAM, Canto Blanco, 28049 Madrid, Spain

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ABSTRACT

The genes encoding the ribosomal P-proteins CcP0, CcP1 and CcP2 of <i>Ceratitis capitata</i> were expressed in the conditional P0-null strains W303dGP0 and D67dGP0 of <i>Saccharomyces cerevisiae</i>, the ribosomes of which contain either standard amounts or are totally deprived of the P1/P2 proteins, respectively. The presence of the CcP0 protein restored cell viability but reduced the growth rate. In the W303CcP0 strain, all four acidic yeast proteins were found on the ribosomes, but in notably less quantity, while a preferable binding of the YP1α/YP2β pair was established. In the absence of the endogenous P1/P2 proteins in the D67CcP0 strain, the complementation capacity of the CcP0 protein was considerably reduced. The simultaneous expression of the three medfly genes resulted in alterations of the stalk composition: both the CcP1 and CcP2 proteins were found on the particles substituting the YP1α and YP2α proteins, respectively, but their presence did not alter the growth rate, except in the case of the YP1α/β defective strain, where a helping effect on the binding of the YP2α and YP2β proteins on the ribosomes was confirmed. Therefore, the medfly ribosomal P-proteins complement the yeast P-protein deficient strains forming an heterogeneous ribosomal stalk, which, however, is not functionally equivalent to the endogenous one.

INTRODUCTION

Insight into the structure of the ribosomes and their components at high resolution leaves no question that the architecture of the translational machinery of the cell has been strongly conserved in all kingdoms. However, inter-kingdom differences among ribosomal components inevitably exist, although the functional significance of these structural variations has not been clarified yet. The challenge to explore possible functions of non-conserved ribosomal features, as well as to understand how the same structural landmarks on evolutionary divergent ribosomes delineate strongly conserved functional domains, has led to the <i>in vivo</i> functional studies of protein synthesis constituents in eucaryotic heterologous systems (1–3).

One of the most characteristic ribosomal structures is the stalk: a highly flexible and universal lateral protuberance on the large subunit which is directly involved in the interaction of elongation factors, participating in the translocation mechanism (4). In eucaryotes the stalk is formed by the pentameric complex P0–(P1)2(P2)2 that is reminiscent of the bacterial complex L10–(L7/L12)4. In particular, the P0 protein is the eucaryotic L10 equivalent (5) and has a key role in the stalk structure. This protein binds to the highly conserved 26S/28S rRNA GTPase center through the N-terminal domain (6) at sites that are equivalent to those found in bacteria (7). Moreover, the P0 C-terminal domain interacts with the acidic phosphoproteins P1 and P2 (the L7/L12 equivalents) through their N-terminal domains, forming the tip of the stalk (8).

The principal difference between the individual stalk components of the 80S and 70S ribosomes is that the procaryotic acidic protein is genetically unique, whereas their eucaryotic counterparts are encoded by several independent genes. Two types of P-proteins, called P1 and P2, have been described in mammals and insects (9), while the number is higher in some lower eucaryotes, especially in plants and protozoa. In the yeast <i>Saccharomyces cerevisiae</i> four different P-proteins have been found; two of them belonging to the P1 family (YP1α and YP1β) and two to the P2 family (YP2α and YP2β), based on amino acid sequence comparisons to their mammalian counterparts (for a review see 10). In the medfly <i>Ceratitis capitata</i>, biochemical analysis of total ribosomes revealed that the ribosomal P-protein family consists of two acidic proteins, CcP1 and CcP2, of 17 and 15 kDa, respectively, and of one 34 kDa basic protein, CcP0 (11,12). The genes encoding these proteins have been recently cloned and identified by genomic and
cDNA sequence analysis and the encoded polypeptides show considerable similarity with the yeast proteins (12,13).

Our understanding on the molecular mechanisms, which support a central role of the stalk structure in the regulation of the ribosomal activity, has undergone remarkable changes during the recent years. Genetic analysis on *S.cerevisiae* revealed that the P1 and P2 proteins are not essential for cell viability, despite the fact that they are required for certain aspects of growth and development (14). The four yeast P1 and P2 proteins have not identical functional roles, as they cannot substitute each other. In fact, the simultaneous presence of the P1 and P2 proteins is required for in vivo binding on the ribosomes (15). Interestingly enough, yeast ribosomes lacking the P1 and P2 proteins selectively translate a different subset of mRNAs than ribosomes containing these proteins, both in vitro and in vivo (14). P-protein-depleted ribosomes seem to exist in the cell and their number increases in certain metabolic conditions (16). On the contrary, the P0 protein is absolutely required for yeast viability, suggesting that this protein by itself provides the minimal stalk structure indispensable for ribosome function (17).

In this context, the reason for the existence of more than two acidic proteins in a number of eucaryotic species is not immediately evident. A higher complexity of the stalk may indicate a more complicated mechanism for the regulatory process in which this ribosomal structure may be involved (10). Studying the functional relationship between the four proteins from yeast and the two proteins from *C.capitata* may provide experimental bases to understand the reasons for the stalk complexity. Therefore, we considered it interesting to express the genes of the medfly P-protein family, CcP0, CcP1 and CcP2, in the conditional P0-null strains, W303dGP0, which contains standard amounts of four P1/P2 proteins (17) and D67dGP0 which is totally deprived of these proteins (8).

**MATERIALS AND METHODS**

**Yeasts and bacterial strains and growth media**

*Saccharomyces cerevisiae* W303dGP0 (MATα, leu2-3, 112, ura3-1, trp1-1, his3-11, 15, ade2-1, can1-100, rpP0:URA3-GAL1-rpP0) and D67dGP0 (MATα, leu2-3, 112, ura3-1, trp1-1, his3-11, 15, ade2-1, can1-100, rpYP1:LEU2, rpYP1β:TRP1, rpP0:URA3-GAL1-rpP0) strains were derived from W303 and D67 (15), respectively, by integration through homologous recombination, in the rpP0 locus, of a construction carrying the P0 coding region fused to the GAL 1 promoter (8,17). Yeasts were grown at 30°C in either YEP medium (1% yeast extract, 2% peptone) or minimal YNB medium, supplemented with the necessary nutritional requirements. In both cases, the carbon source was either 2% glucose or 2% galactose, as indicated. *Escherichia coli* DH5α was used as a host for the routine maintenance and preparation of plasmids and was grown in LB medium.

**Plasmids**

The transformation of the yeast mutant strains with the genes CcP1, CcP2 and CcP0 of the medfly *C.capitata* was made with the plasmids pFL37 and prSAH I. pFL37 was derived from pFL38 (18), by removing the URA3 marker with *Bgl*II and by introducing in the same position a *Bam*HI fragment carrying the HIS3 marker. The prRSA plasmid was derived from the prRSA plasmid, by addition of the hygromycin resistance marker. pFL37 CcP0 was derived from pFL37 by introducing a 2700 bp *Hinc*II-*EcoRI* fragment containing the coding region of the CcP0 cDNA of *C.capitata*, flanked by the 5′ and 3′ regulatory regions of the YP0 protein of *S.cerevisiae* of 1015 and 834 bp, respectively. A 971 NdeI–NheI fragment with the coding sequence of the CcP0 cDNA was initially introduced in the corresponding sites of the plasmid pBSP0 (8), carrying the coding region, as well as the 5′ and 3′ flanking regions of the yeast YP0 protein. The NdeI and NheI restriction sites, respectively, are shown in bold. pFL37 CcP0P1 was derived from pFL37CcP0, by introducing a 2095 bp *EcoR*I–*Bam*HI fragment containing the coding region of the CcP1 cDNA of *C.capitata*, flanked by the 5′ and 3′ regulatory regions of the YP1α protein of *S.cerevisiae* of 562 and 1200 bp, respectively. An NdeI fragment of 353 bp with the coding sequence of the CcP1 cDNA of *C.capitata* was initially introduced in the corresponding sites of the plasmid pBSYP1α, carrying the 5′ and 3′ flanking regions of the YP1α protein of *S.cerevisiae*. The NdeI restriction site was introduced at both ends of the CcP1 gene by PCR, using as template plasmid pBSCP0 DNA (13) and the synthetic oligonucleotide primers TAACCcP1 (5′-ACAAAAACGCAATATGTTAATCGAGAGAC-3′) and M13F-40 (Stratagene). The NdeI restriction site is shown in bold. pFL37 CcP0P2 was produced similarly to pFL37 CcP0CcP1, using as template for the PCR reaction plasmid pBSCP2 DNA (13), carrying a 2.5 kb DNA genomic fragment with the CcP2 gene of *C.capitata* and the synthetic oligonucleotide primers ATGCCcP2 (5′-TTTCCCTGCGCATATGGTTACGGAGAC-3′) and TAACCcP2 (5′-GATTTAGCATAATTCTTACGAGAGAC-3′). pRSAHCCcP2 was derived from pRSAH I, by introducing a 2101 bp *EcoR*I–*Bam*HI fragment carrying the coding region of the CcP2 gene, flanked by the 5′ and 3′ regulatory regions of the yeast YP1α protein, in a *Sma*I site.

**Ribosome extraction, electrophoretical methods and western blot analysis**

Total purified ribosomes were isolated from yeast strains grown in glucose and from six-day larvae of *C.capitata* as previously described (11,19). SDS-electrophoresis in 15% acrylamide slab gels and isoelectrofocusing were carried out as described (11,20). Briefly, ribosomes treated with RNase A (10 mg/ml) were lyophilized, resuspended in loading buffer (6% ampholytes, 8 M urea) and directly loaded in a standard vertical gel (5% acrylamide, 0.2% bis-acrylamide, 6 M urea, 6% ampholytes pH 2.5–5). Samples were overloaded with 2 M urea, while 30 mM NaOH and 180 mM H2SO4 were used as upper and lower buffers, respectively. Gels were either silver stained or electro-transferred to nitrocellulose membrane and probed with the monoclonal antibody 3B6H5, specific to the highly conserved C-terminus of eucaryotic acidic proteins (21). The IgGs were localized with peroxidase-labeled second
antibody (RaM/PO) and detected by chemiluminescence, using the ECL system (Amersham).

**Peptide sequencing**

Proteins in PVDF membranes were sequenced by Edman degradation in a 447 automatic peptide sequenator (Applied Biosystems) at the Centro de Biología Molecular Protein Sequencing Service.

**RESULTS**

**Determination of the *C.capitata* P0-protein complementation capacity in *S.cerevisiae* conditional P0-null strains**

The plasmids pFL37CcP0, pFL37CcP0P1, pFL37CcP0P2 and pRSAHCcP2, carrying the genes encoding the ribosomal P proteins, CcP0, CcP1 and CcP2 of the medfly *C.capitata*, were used for the transformation of the *S.cerevisiae* conditional null mutants W303dGP0 and D67dGP0, expressing the genomic P0 gene only in galactose (8,17). The D67dGP0 is, additionally, an acidic protein-defective strain with the YP1α/β genes inactivated by disruption, and the ribosomal stalk, totally deprived of acidic proteins, consists only of the P0 protein (15). The transformed strains were grown in glucose, either in agar plates or in liquid medium, to test the capacity of the medfly P-protein genes to restore cell growth. All the strains, except D67dGP0 with the CcP0 gene alone (D67CcP0) or together with the CcP2 gene (D67CcP0P2), were able to grow in solid medium (not shown), indicating that the heterologous CcP0 protein was expressed and restored viability. In liquid medium, strains W303CcP0, W303CcP0P1, W303CcP0P2 and W303CcP0P1P2 presented exponential doubling times of 135, 144, 120 and 141 min, respectively (Table 1). Interestingly, the strains D67CcP0 and D67CcP0P2 that were unable to grow on plates, supported very slow growth in liquid only in the absence of agitation. The introduction of the CcP1 gene in these two slow-growing strains restored the aerobic growth in glucose liquid medium, with a doubling time of 166 (D67CcP0P1) and 183 min (D67CcP0P1P2). In the same conditions, the control strains transformed with the endogenous P0 protein gene, W303YP0 and D67YP0, had a doubling time of 95 and 255 min, respectively.

**Stalk composition in ribosomes from strains W303CcP0 and D67CcP0 transformed with CcP0**

The presence of the heterologous CcP0 protein on the ribosomes of the transformed strains W303CcP0 and D67CcP0 was detected by isoelectrofocusing in a 3.0–10.0 pH gradient and immunoblotting with the monoclonal antibody 3BH5, specific to the C-terminus of eucaryotic ribosomal P proteins. Total purified ribosomes from each glucose-grown strain were analyzed, while ribosomes from *S.cerevisiae* W303YP0 and from *C.capitata* six-day larvae were used as controls. The endogenous yeast P-proteins appeared as one band corresponding to the YP0 protein, and one more acidic band, corresponding to the acidic proteins YP1α/β and YP2α/β (Fig. 1, lane 1), while those of the medfly appeared as one basic band, corresponding to the CcP0 protein, and two more acidic bands, corresponding to the acidic proteins CcP2 and CcP1, respectively (Fig. 1, lane 4). The ribosomes of the strain W303CcP0 (Fig. 1, lane 2) showed the medfly CcP0 protein band and the YP1α/β and YP2α/β band, while no YP0 band was present. One minor band at the same pH area with CcP0 might represent a post-translational modification of the CcP0 protein. Similarly, the D67CcP0 ribosomes showed only the

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Table 1. Growth of *S.cerevisiae* conditional P0-null mutants, transformed with plasmids encoding the proteins CcP0, CcP1 and CcP2 of *C.capitata*

In the same conditions, the control yeast strains W303YP0 and D67YP0, transformed with the gene of the endogenous YP0 protein, presented a doubling time of 95 and 255 min, respectively.

*Strains D67CcP0 and D67CcP0P2 grow very slowly in glucose liquid medium, at 30°C, in the absence of agitation.*
isoelectrofocusing, in vertical 5% acrylamide gel, in the presence of ampholines pH 5.0 (top) to 2.5 (bottom). The gel was silver stained. Ribosomes from the W303YP0 strain (Fig. 3A, lane 1) and both 17 and 15 kDa bands in the W303CcP0P1P2 samples and to the yeast YP0 protein in the W303YP0 strain. Both proteins have the same molecular weight, but different pIs (Fig. 1). As shown previously (Fig. 1), no YP0 protein is present in S.cerevisiae transformants expressing CeP0.

In the case of the S.cerevisiae D67 transformants, Figure 3B illustrates that, besides the CeP0 34 kDa band, a CeP1 17 kDa band was found in strain D67CcP0P1 (Fig. 3B, lane 4) and both CeP1 17 kDa and CeP2 15 kDa bands were found in D67CcP0P1P2 (Fig. 3B, lane 6). In both strains, a 12 kDa band, corresponding to the yeast YP0 protein, was also detected, implying a helping effect in their binding by the heterologous CeP1 protein. On the contrary, no acidic protein band was detected on the ribosomes of the transformed strains D67YP0 or D67CcP0P2 (Fig. 3B, lanes 2 and 3, respectively). Confirming previous results (15), in the absence of P1 proteins, either homologous or heterologous, no P2 protein could bind on the ribosomes.

Acidic protein alterations in the ribosomal stalk of yeast strains expressing the medfly P-proteins

The stalk acidic protein composition was analyzed by 2.5–5.0 pH gradient isoelectrofocusing. Ribosomes from C.capitata six-day larvae and from S.cerevisiae W303YP0 were used as controls (Fig. 4, lanes 1 and 2).

Simultaneous expression of CeP0 and CeP1 (Fig. 4A). The W303CcP0P1 ribosomes contained the yeast acidic proteins, YP2α, YP2β and YP1β, but not YP1α, and, in addition, two higher pI bands (~4.0) were present (Fig. 4A, lane 4). Edman degradation sequencing of these two bands confirmed that, albeit having a more basic character than the native protein (Fig. 4A, lane 1), they correspond to CeP1. Moreover, the two bands were reduced to only one of higher pI upon alkaline phosphatase treatment of the W303CcP0P1 ribosomes (data not shown), implying that CeP1 could be phosphorylated in yeast, as the native protein (11). Its more basic character, when expressed in yeast, might be due to an additional, post-translational

and YP2α. This pattern was reproducible, even after a second centrifugation of the ribosomes on sucrose gradient.

SDS-electrophoresis of stalk proteins in the ribosomes of the S.cerevisiae strains expressing the proteins CeP0, CeP1 and CeP2

The medfly ribosomal P-proteins CeP0, CeP1 and CeP2 were detected on the ribosomes of the transformed yeast strains W303CcP0P1, W303CcP0P2 and W303CcP0P1P2 by SDS electrophoresis in 15% acrylamide gel and immunoblotting. As shown in Figure 3A, besides the 12 kDa yeast acidic protein band, an additional CeP1 17 kDa band was present in the ribosomes of the W303CcP0P1 strain (Fig. 3A, lane 3), a CeP2 15 kDa band in those from strain W303CcP0P2 (Fig. 3A, lane 4), and both 17 and 15 kDa bands in the W303CcP0P1P2 particles (Fig. 3A, lane 5). The amount of CeP2 seems to be lower than the amount of CeP1, as previously shown for the Dicystostelium discoideum proteins (3). This can be due to either a higher degradation of this protein in the yeast cell or to a lower level of expression. These bands were not present in the ribosomes of the control W303YP0 (Fig. 3A, lane 1) and W303CcP0 (Fig. 3A, lane 2) strains. The 34 kDa band corresponds to the C.capitata CeP0 protein in the W303CcP0, W303CcP0P1, W303CcP0P2 and W303CcP0P1P2 samples and to the yeast YP0 protein in the W303YP0 strain. Both proteins have the same molecular weight, but different pIs (Fig. 1). As shown previously (Fig. 1), no YP0 protein is present in S.cerevisiae transformants expressing CeP0.

For better resolution of the yeast acidic proteins ribosomes were also analyzed by isoelectrofocusing in a 2.5–5.0 pH gradient. All four yeast acidic proteins YP1α, YP1β, YP2α and YP2β were detected on ribosomes from W303CcP0 (Fig. 2, lane 2), but in remarkably lower quantity compared to the W303YP0 ribosomes (Fig. 2, lane 1). Interestingly, YP1α and YP2β were found in much higher quantities than YP1β

CcP0 band (Fig. 1, lane 3), while neither YP0 nor any acidic proteins were present.

Figure 2. Isoelectrofocusing of ribosomes from the yeast strain W303CcP0 (lane 2), grown in glucose medium. Total ribosomes (300 µg), subjected to two sequential centrifugations through two steps of sucrose, were resolved by isoelectrofocusing, in vertical 5% acrylamide gel, in the presence of ampholines pH 10.0 (top) to 3.0 (bottom), blotted to nitrocellulose membrane and incubated with the monoclonal antibody 3HB5. Fifty micrograms of ribosomes from the yeast strain W303YP0 (lane 1) and six-day larvae of C.capitata (lane 4) were used as control. Positions of the proteins CcP0, CeP1 and CeP2 of C.capitata and YP0, YP2α/β and YP1α/β of S.cerevisiae are indicated.

Figure 1. Immunoblotting of ribosomes from the yeast strains W303CcP0 (lane 2) and D67CcP0 (lane 3) grown in glucose medium: 50 µg of treated with RNase A ribosomes were resolved in vertical, 5% acrylamide gel, in the presence of ampholines pH 10.0 (top) to 3.0 (bottom), blotted to nitrocellulose membrane and incubated with the monoclonal antibody 3HB5. Fifty micrograms of ribosomes from the yeast strain W303YP0 (lane 1) and six-day larvae of C.capitata (lane 4) were used as control. Positions of the proteins CcP0, CeP1 and CeP2 of C.capitata and YP0, YP2α/β and YP1α/β of S.cerevisiae are indicated.
The presence of acidic bands corresponding to both the medfly CcP1 and the yeast YP2α/β proteins was also established on the ribosomes of the YP1α/β deficient yeast strain D67CcP0P1 (Fig. 4A, lane 5), confirming that the medfly CcP1 protein was able to form part of the P-protein stalk complex, interacting with the CcP0 protein and helping the binding of both yeast YP2α/β proteins.

Simultaneous expression of CcP0 and CcP2 (Fig. 4A and B). In W303CcP0P2, the stalk consisted mainly of the endogenous YP2β and YP1α proteins with very small quantities of YP2α and YP1β; a pI 4.5 band, that must correspond to medfly CcP2 protein, was also detected (Fig. 4A, lane 3). CcP2 is usually very faintly stained with silver, even in the control medfly ribosomes (Fig. 4A, lane 1), so its presence in the ribosomes was verified by immunoblotting with the monoclonal antibody 3HB5. Ribosomes from the yeast strain W303YP0 (A and B, lane 1) and D67YP0 (B, lane 2) were used as control. Positions of the proteins CcP0, CcP1 and CcP2 of C.capitata and YP0, YP2α/β and YP1α/β of S.cerevisiae are indicated.
proteins, CcP1 and CcP2, and the yeast proteins YP2β and YP1α. Traces of YP2α and YP1β are also detected (Fig. 5, lane 2). The identity of the faintly stained CcP2 protein was again verified by immunoblotting (Fig. 5, lane 4). The presence of the YP1α protein, which in the double transformed strain W303CcP0 was replaced by CcP1, is believed to be due to a preferential formation of a YP2β/YP1α heterodimer.

On the other hand, the ribosomes of the D67CcP0P1P2 strain, defective of the YP1α/β proteins, contained only the YP2β protein (Fig. 5, lane 1) besides the medfly proteins CcP1 and CcP2.

DISCUSSION

We have performed an in vivo functional analysis, expressing the ribosomal P-protein genes of the medfly C. capitata in the S. cerevisiae P0 conditional null mutants W303dGP0 and D67dGP0. Their ribosomes contain either standard amounts of the four acidic proteins YP1α/β and YP2α/β (W303dGP0) or are totally deprived of acidic proteins (D67dGP0). A summary of the results is shown in Figure 6. The proposed models show the acidic protein distribution in the ribosomal stalk structure of the yeast strains expressing the medfly P-proteins, including (i) substitution of the endogenous acidic proteins YP1α and YP2α by the heterologous CcP1 and CcP2, respectively; (ii) preferential binding of the YP1α/YP2β proteins to the heterologous CcP0 protein and (iii) a helping effect of the heterologous CcP1 protein in the binding of the endogenous YP2α/β proteins, on the ribosomes of the YP1α/β defective strains.

The expression of the CcP0 protein, under the control of the 5’ and 3’ regulatory regions of the yeast YP0 gene, in the transformed strain W303CcP0 allowed cell growth at restrictive conditions, namely in glucose medium, but the growth rate was slower than with the homologous P0 protein. Similar results have been recently shown for the Aspergillus fumigatus, D. discoideum and Rattus norvegicus P0 genes, but not for Leishmania infantum (Rodriguez-Gabriel et al., unpublished results). Further analysis of the W303CcP0 ribosomes revealed a preferential binding of the YP1α/YP2β proteins to the heterologous CcP0 protein, also noticed for the P0 proteins of other species (Rodriguez-Gabriel et al., unpublished results). It seems that these two proteins show a higher affinity for the heterologous CcP0 than YP1β/YP2α, suggesting that they are evolutionarily closer to CcP1 and CcP2. Alternatively, YP1α and YP2β may be less dependent on P0 for binding to the ribosome and, therefore, are less affected by the presence of a heterologous protein. Supporting this possibility it has been found that in yeast ribosomes lacking L12, a protein also involved in the stalk structure, only proteins YP1β/YP2α are bound while YP1α/YP2β are released from the ribosomes (22). Independently of the conclusions regarding the interaction with the medfly P0, all these results indicate a preferential association of the four yeast acidic proteins in two heterodimers YP1α/YP2β and YP1β/YP2α, which may play the role of the P1 and P2 homodimers found in other eucaryotes.

In the absence of ribosome bound yeast acidic proteins, the medfly CcP0 protein is considerably less efficient in complementing yeast growth. Thus, the D67dGP0 mutant grows in liquid glucose medium very slowly and only in almost anaerobic conditions, and does not grow on glucose agar plates. Possibly, the ribosomes carrying the medfly P0 interact poorly with the elongation factors in the absence of yeast acidic proteins. In these conditions, the pattern of protein expression can be altered, affecting the cell growth. Different yeast growth phenotypes have been reported, as a result of alterations on the stalk composition (19,23). These results suggest, in addition, that the ability of the heterologous P0 proteins to support cell growth in yeast is related to their capacity to bind the endogenous acidic proteins in the ribosomal stalk.

The composition of the yeast stalk was clearly altered when the CcP1 and CcP2 proteins were introduced in yeast together with CcP0. In the W303CcP0 strain, the introduction of the CcP1 gene resulted in the formation of an unusual stalk: the CcP1 protein was bound to the CcP0 protein together with the endogenous proteins YP2β, YP2α and YP1β. It seems that CcP1 substitutes YP1α, which is missing from the ribosomes. This is in agreement with a closer structural relationship between YP1α and CcP1 previously commented. However, the presence of the heterologous CcP1 protein on the particles did not have significant influence on the cell growth, which was slightly reduced compared to the W303CcP0 strain. In contrast, CcP1 had a strong positive effect on the yeast growth in the D67 background. Thus, the introduction of the CcP1 gene restored normal growth in glucose of the anaerobic, slow-growing D67CcP0 strain. In the D67CcP0P1 ribosomes, CcP1 was also bound and, in addition, promoted the binding of the YP2α/β proteins, which were responsible for the increase of cell growth.

The introduction of the CcP2 protein in transformants expressing the CcP0 protein had different influence than CcP1.
In the W303CcP0 ribosomes, the presence of the heterologous CcP2 protein did not affect the binding of the proteins YP1α and YP2β, in contrast to YP1β and YP2α which were drastically reduced. The CcP2 protein seemed to replace the YP2α protein and in its absence, the YP1β protein could not bind either, confirming the existence of the previously mentioned YP1α/YP2β and YP1β/YP2α associations. Interestingly enough, the presence of the CcP2 slightly increased the cell growth, a fact that might be due to a stabilizing effect of the medfly protein on the binding of YP1α/YP2β to CcP0. On the contrary, the introduction of the CcP2 gene in the D67CcP0 strain did not affect either the strain growth or the composition of the stalk; the D67CcP0CcP2 ribosomes were still totally depleted of acidic proteins, either endogenous or heterologous. This is in agreement with previous data indicating that the presence of at least one member of the P1 protein group, either from S. cerevisiae or C. capitata, is indispensable for the binding of the CcP2 protein on the yeast ribosomes. Confiming the replacement of YP2α by CcP2 in the D67CcP0P1P2 strain, only the YP2β protein was present on the ribosomes apart from the two medfly acidic proteins. This substitution had, however, a negative effect on growth confirming that CcP2, although structurally equivalent to YP2α, is not functional in yeast.

Apart from establishing a structural relationship between yeast YP1α and CcP1 on one side and YP2α and CcP2 on the other, these results also reinforce the existence of a specific function for each one of the four yeast acidic proteins. Moreover, they indicate that the ribosomal stalk is a structural and functional unity which has been forced, during the evolution, to maintain unaltered some features essential for cell viability. Thus, the amino acid moiety of the rRNA binding domain in the N-terminal region of the P0 protein has been highly conserved, supporting the conserved yeast RNA binding capacity of the CcP0 protein. Likewise, the C-terminal peptide is almost identical in all the members of the P-protein family both in the medfly and in yeast. However, this is not, by itself, sufficient for protein complementation, and in the absence of the yeast acidic proteins, the CcP0 protein cannot restore the cell growth at restrictive conditions. Similarly, the binding of either the CcP1 or CcP2 protein on the particles, substituting the endogenous acidic proteins in strain W303, did not have any considerable positive effect on the growth rate. Additionally, the substitution of the YP2α protein by CcP2 in the D67CcP0CcP2 ribosomes resulted in a reduction of the growth rate, showing clearly that there is no functional equivalence between the CcP2 and the YP2α proteins, in spite of their similar C-end. On the contrary, the helping effect of the CcP1 protein on the binding of the YP2α/YP2β proteins to the ribosomes of the YP1 protein (defective D67CcP0P1 strain) caused a strong increase of the growth rate.

It seems therefore, that besides the conserved C-terminal peptide, other structural elements of the P-proteins participate in the interaction of the elongation factors with the ribosomes. These regions have notably evolved, as did the P0 region that contributes to the formation of the pentameric complex with the P1 and P2 proteins, leading to an incompatibility of the P-proteins from different eucaryotic species.

**Figure 6.** Proposed models of the endogenous and heterologous acidic P-protein distribution in the ribosomal stalk structure of transformed yeast strains, expressing the medfly CcP0, CcP1 and CcP2 proteins, and of the control strains W303YP0 (wild-type) and D67YP0 (mutant), respectively. The doubling times in glucose medium are also indicated. Proteins in small quantity are shown in light gray (strains W303CcP0 and W303CcP0P2). Although the protein interactions are not experimentally confirmed, the scheme is compatible with the results. The C. capitata P1/P2 proteins are present as monomers in the hybrid ribosomes, forming a heterodimer (CcP1/CcP2). These data open up the possibility for the existence of two heterodimers (P1/P2)(P1/P2), rather than dimers (P1/P1)(P2/P2) in higher eucaryotic ribosomes. *Strains D67CcP0 and D67CcP0P2 grow very slowly in glucose liquid medium, at 30°C, in the absence of agitation.
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