Phosphorylation of Ribosomal Protein P0 Is Not Essential for Ribosome Function but Can Affect Translation†

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ABSTRACT: Protein P0, an essential component of the eukaryotic ribosomal stalk, is found phosphorylated in the ribosome. Substitution of serine 302 in the amino acid sequence of the *Saccharomyces cerevisiae* P0 by either aspartic acid or cysteine abolishes in vitro and in vivo phosphorylation of the protein. On the contrary, the replacement of this serine by a threonine results in an increase in the protein phosphorylation under both sets of conditions. Therefore, this serine residue, which is part of a consensus casein kinase II modification site, SDDD, seems to be the phosphorylation site in protein P0. The effect of the mutations on the protein activity has been tested in *S. cerevisiae* W303dGP0 and D67dGP0, both of which carry a genomic P0 gene under the control of the GAL1 promoter. Transformation of the mutated genes in *S. cerevisiae* W303dGP0 allows cell growth at 30 °C in glucose—to repress the wild-type P0 expression—at the same rate as controls, and the ribosomes contain a normal amount of the other stalk components. A similar absence of effect of the mutations on growth was found in strain D67dGP0, which has ribosomes deprived of the P1 and P2 proteins. Therefore, P0 phosphorylation is not a requirement for ribosome activity in standard growth conditions either in the presence or in the absence of the other stalk proteins. However, a phenotypic effect is detected in the case of strain D67 transformed with the overphosphorylated threonine containing P0, which contrary to the wild-type and the other mutated proteins is unable to support cell growth at 37 °C in the presence of either 0.3 M NaCl or 0.8 M sorbitol. In vitro polymerizing tests indicate that this effect is not due to the thermosensitivity of the mutated protein. The results indicate that although P0 phosphorylation is not required for the overall ribosome activity, it may affect the expression of specific proteins involved in metabolic processes such as osmoregulation.

Protein phosphorylation is usually a signal pointing to the involvement of the modified polypeptide in some regulated cellular process. Contrary to the bacterial ribosome, a number of proteins are found phosphorylated on the eukaryotic particles, and not surprisingly they have been considered as potential regulators of the ribosome activity. In the case of protein S6, whose phosphorylation level is affected by the metabolic conditions of the cell, there is considerable experimental evidence supporting its involvement in a translational control mechanism (see ref *1* for a recent review). Interestingly, *Saccharomyces cerevisiae* is an exception, probably because the yeast S6 lacks the serines whose phosphorylation is involved in the control mechanism (*2*).

Protein P0 and the acidic proteins P1 and P2 are also found phosphorylated in the ribosome. These proteins form a pentameric complex ($P0-P1_2-P2_2$) that composes the eukaryotic ribosomal stalk (*3*), a highly flexible universal structure involved in the interaction of the elongation factors with the ribosome during protein synthesis (*4*). An exchange between the phosphorylated P1 and P2 proteins in the ribosome and in a relatively large cytoplasmic pool of nonphosphorylated polypeptides has been reported (*5*−*7*). This has led to the proposal that these ribosomal components are involved in a possible ribosome regulatory mechanism (*8*).

Protein P0 has a central role in the stalk structure. This protein binds through the N-terminal domain to a highly conserved region of the 26S/28S rRNA, the so-called GTPase center (*9*), while its carboxyl domain interacts with proteins P1 and P2, forming the tip of the stalk (*10*). The three proteins, P0, P1, and P2, share the same C-terminal peptide, ESDDDMGFGLFD, which is highly conserved in the equivalent ribosomal proteins, from yeast to humans (*8*). In *S. cerevisiae*, P1- and P2-type proteins are not essential for ribosome activity, and protein synthesis takes place in vivo as well as in vitro in the absence of these proteins, though at a lower rate (*11*). It seems, therefore, that P0 by itself provides the minimal stalk structure indispensable for ribosome function.

Phosphorylation of P1 and P2 proteins takes place at the serine residue in the conserved C-terminal peptide (*12*−*14*), and it has been reported to be required for in vitro reconstitution of active core ribosomal particles deprived of these proteins by various methods in different systems (*15*−

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17). Nevertheless, the mutation of the phosphorylatable serine residue in *S. cerevisiae* acidic proteins has been shown not to have an effect in vivo on either their interaction with the ribosome or their function, since the mutant strains grow at the same growth rate as the controls (*14*).

One possible explanation for the apparent discrepancy between the in vivo and in vitro results regarding the role of P1 and P2 phosphorylation could be the presence of a phosphorylated P0 protein that was not taken into account in the previous experiments. The central role that P0 plays in the stalk structure and function makes it conceivable that its phosphorylation may overcome the effect that the P1 and P2 mutations have in the in vivo tests. In addition, since the protein P0 alone supplies the minimal structure necessary for ribosome activity, it is possible to study the role of phosphorylation on the stalk activity in the absence of the P1 and P2 proteins.

The phosphorylation site of protein P0 has not been experimentally located. However, the presence in P0 of the same carboxyl end that is phosphorylated in P1 and P2 strongly suggests the equivalent serine might also be modified in this protein. To confirm this possibility and study the role of P0 phosphorylation, a mutational analysis of serine in position 302 in the C-terminal end of the *S. cerevisiae* protein was carried out and the results are presented here.

MATERIALS AND METHODS

Yeast and Bacterial Strains. *S. cerevisiae* W303-1b (MATα *leu2-3,112 trp1-1 ura3-1 his 3-11,15 ade2-1 can1-100*) and *S. cerevisiae* D67 (α *leu2-3,112 trp1-1 ura3-1 his 3-11,15 rpYP1α::LEU2 rpYP1β::TRP1*) were derived from W303 by gene disruption (Remacha et al., 1992). *S. cerevisiae* W303dGP0 (MATα *leu2-3,112 trp1-1 his 3-11,15 ade2-1 can1-100 rpP0::URA3-GAL1-rpP0*) and *S. cerevisiae* D67dGP0, (α *ura3-1 his3-11,15 rpYP1α::LEU2 rpYP1β::TRP1 rpP0::URA3-GAL1-rpP0*) were obtained from *S. cerevisiae* W303-1b and D67, respectively, and they carry the P0 coding region under the GAL1 promoter (*25*). The yeast were grown in either rich YEP medium (1% yeast extract and 2% peptone) or minimal SD medium (*18*) supplemented with the necessary nutritional requirements. In both cases either 2% glucose or 2% galactose was used as carbon source as indicated.

Escherichia coli DH5α was used as the host for the routine maintenance and preparation of plasmids used in these studies and was grown in LB medium.

Enzymes and Reagents. Restriction endonucleases were purchased from Boehringer Mannheim GmbH, New England Biolabs, Inc., and Amersham Corp. and were used as recommended by the suppliers. T4 DNA ligase, calf intestinal alkaline phosphatase, and the DNA polymerase I Klenow fragment were from Boehringer Mannheim, and DNA polymerase I and T4 DNA polymerase were from New England Biolabs. [γ-^{32}P]ATP and $^{32}PO_4^{3-}$ were obtained from Amersham Corp.

Cell Transformation and Recombinant DNA Techniques. *E. coli* cells were transformed according to ref *19*. *S. cerevisiae* transformations were performed as described by ref *20*.

DNA preparation, restriction enzyme digestions, agarose gel electrophoresis, ligation of DNA fragments, Southern blots, etc., were carried out according to standard techniques (*21*). DNA was sequenced by the dideoxy chain-termination method with universal primers and complementary custom-made oligonucleotides (Isogen).

Table 1: Mutagenic Oligonucleotides[a]

mutation	sequence
cysteine	5′-AAGAAGAAGAAT**G**TGATGACGACAT −3′
cysteine	5′-ATGTCGTCATCA**C**ATTCTTCTTCTT-3′
aspartic acid	5′-AAGAAGAAGAA**A**CTGATGACGACAT-3′
aspartic acid	5′-ATGTCGTCATCAG**T**TTCTTCTTCTT-3′
threonine	5′-AAGAAGAAGAA**GA**TGATGACGACAT-3′
threonine	5′-ATGTCGTCATCA**TC**TTCTTCTTCTT-3′

[a] The two complementary oligonucleotides used for every overlap extension PCR mutagenesis are indicated. The codon for the new amino acid is underlined and the changed nucleotides are in boldface types.

Plasmids. pFL37 was derived from pFL38 (*22*) by removing the URA3 marker with *Bgl*II and inserting in the same site a 1.8 kb *Bam*HI fragment containing the HIS3 marker. BSP0 was obtained by subcloning the yeast P0 gene in the Bluescript KS$^+$ vector (*10*).

Site-Directed Mutagenesis. Serine 302 in the P0 protein amino acid sequence was replaced by either cysteine, aspartic acid, or threonine by site-directed mutagenesis of the P0 gene in plasmid BSP0 by the overlap extension PCR[1] method (*23*). Mutagenic oligonucleotides in Table 1, together with the T7 universal and reverse oligonucleotides, were used as primers. The PCR products were then subcloned as an *Xho*I−*Eco*RI 2.7 kb fragment into the *Sal*I−*Eco*RI sites of plasmid pFL37, producing plasmids pFL37-P0C, pFL37-P0D, and pFL37-P0T. As a control, the wild-type gene was also subcloned into the vector, yielding plasmid pFL37-P0. The mutations in the plasmids were confirmed by DNA sequencing.

Cell Fractionation. Yeasts were grown exponentially in rich YEP medium up to $A_{600} = 1$, and cells were collected by centrifugation and washed with buffer 1 (100 mM Tris-HCl, pH 7.4; 20 mM KCl; 12.5 mM $MgCl_2$, and 5 mM β-mercaptoethanol). Cells in buffer 1 were supplemented with protease inhibitors (0.5 μmol/g PMSF and 1.25 μg/g leupeptin, aprotinin, and pepstatin) and broken with glass beads. The extract was centrifuged in a Beckman SS-34 rotor (12 000 rpm, 15 min, 4 °C), yielding the supernatant S30 fraction, which was afterward submitted to high-speed centrifugation at 90 000 rpm for 30 min at 4 °C in a Beckman TL100.3 rotor. The supernatant S100 fraction was stored at −80 °C and the crude ribosome pellet was resuspended in buffer 2 (20 mM Tris-HCl, pH 7.4, 500 mM NH_4Ac, 100 mM $MgCl_2$, and 5 mM β-mercaptoethanol) and centrifuged through a discontinuous sucrose gradient (20%/40%) in buffer 2 at 90 000 rpm for 120 min at 4 °C in a TL100.3 rotor. The pellet of washed ribosomes was dissolved in buffer 1 and stored at −20 °C. The fraction obtained from the standard S-100 by precipitation between 30% and 70% with ammonium sulfate served as a source of supernatant factors in the polymerizing system.

Protein Analysis. Ribosomal proteins were analyzed either by SDS−PAGE or by isolectrofocusing. Isoelectrofocusing

[1] Abbreviations: SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; ECL, enhanced chemiluminescene; PVDF, poly(vinylidene difluoride); TCA, trichloroacetic acid.

SEQUENCE	N° a.a.	PROTEIN
KYAAAAPAATSAASGDAAPAEEAAAEEEEE**S**DDDMGFGLFD	312	P0
GASSAAAGAAGAAAGGDAAEEEKEEEAKEE**S**DDDMGFGLFD	110	P2ß
AAGPASAGGAAAASGDAAAEEEKEEEAAEE**S**DDDMGFGLFD	106	P2α
VAGAGAASGAAAAGGDAAAEEEKEEEAAEE**S**DDDMGFGLFD	106	P1ß
AAPAGVAGGVAGGEAGEAEAEKEEEEAKEE**S**DDDMGFGLFD	106	P1α

FIGURE 1: Carboxyl ends of the proteins P0, P1α, P1*β*, P2α, and P2*β* are shown. The serine residue found to be phosphorylated in the 12 kDa proteins is marked in boldface type.

was carried out as previously described (*16*), but using whole ribosomes. Particles were pretreated with RNase A (10 *μ*g/mg of ribosomes) on ice for 30−45 min. After lyophilization, the samples were resuspended in loading buffer (6% ampholytes, 8 M urea) and directly loaded into a standard vertical gel (5% acrylamide; 0.2% bisacrylamide, 6 M urea, 6% pH 2.5−5.0 ampholytes). As cathode and anode solutions, 30 mM NaOH and 180 mM H_2SO_4 were used at the top and bottom parts of the gel, respectively. Isolectrofocusing was run in the cold room at 6 mA constant current until the voltage reached 600 V and then at 250 V for 16 h.

Proteins were usually detected by standard silver staining. Alternatively, gels were stained in a solution containing 0.25% Coomassie BB R-250 (Sigma) dissolved in 45% ethanol/10% acetic acid. After 30 min, gel was destained in the same solution but without stain.

Western Blotting. Proteins in gels were transferred to nitrocellulose membranes by electrophoresis in a semidry system with Novablot LKB buffer. The membranes were treated with 5% nonfat milk dissolved in TBS (10 mM Tris-HCl, pH 7.4, and 200 mM NaCl) for 30 min, and afterward they were incubated for 1 h with the antibody diluted in the same buffer. Subsequently, the membranes were washed 15 min in TBS containing 5% nonfat milk and 0.1% Tween 20, and the second antibody (RαM/PO or GαR/PO, from Nordic, Tilburg, The Netherlands), diluted in the former buffer, was added and the sample was incubated for 30 min. Finally, the membrane was washed 15 min with 0.1% Tween 20 in TBS. Bound antibodies were located by detecting peroxidase activity with the ECL system (Amersham) and then the blot was exposed to film.

Protein Phosphorylation. Proteins were phosphorylated in vivo by adding 1 mCi of $^{32}P0_4^{3-}$ to mid-log-phase cells growing in 100 mL of a glucose/low-phosphate medium (*24*). Cells were allowed to grow for 90 min, collected by centrifugation, and processed for preparation of ribosomes as usual.

In vitro phosphorylation was performed by incubating ribosomes (50 *μ*g) in 15 *μ*L of 20 mM Tris-HCl, pH 7.4, 15 mM $MgCl_2$, 80 mM KCl, 6 mM 2-mercaptoethanol, and 60 mM ATP at room temperature for 25 min. The reaction was initiated by the addition of 0.2 *μ*Ci of [*γ*-^{32}P]ATP. After incubation, the samples were supplemented with 15 *μ*L of loading buffer and directly resolved by SDS−PAGE.

Peptide Sequencing. Proteins in PVDF membranes were sequenced by Edman degradation in an Applied Biosystems 447 automatic peptide sequenator at the Centro de Biologia Molecular Protein Sequencing Service.

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 poly(uridylic acid), 40 *μ*M [^{3}H]phenylalanine (212 cpm/pmol), 0.5 mM GTP, 1 mM ATP, 2 mM phosphocreatine, and 40 *μ*g/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

Mutation of Serine 302 in S. cerevisiae P0 Protein. The *S. cerevisiae* stalk ribosomal proteins P0, P1α, P1*β*, P2α, and P2*β* share the same highly conserved C-terminal peptide (Figure 1), which is also present in the equivalent proteins from most species (*8*). The serine residue in this peptide has been shown to be the phosphorylation site in the 12 kDa acidic proteins P1 and P2 from different organisms including yeast (*12*−*14*). The 34 kDa protein P0 is also found phosphorylated in the ribosome, but its site of modification has not been investigated. To test whether the serine in the P0 carboxyl end, corresponding to position 302 in the yeast protein, is also the site of phosphorylation in this polypeptide it was substituted by either cysteine, aspartic acid, or threonine.

The plasmids pFL37-P0C, pFL37-P0D, and pFL37-P0T, carrying the mutated genes, as well as pFL37-P0 containing the wild-type gene, were used to transform *S. cerevisiae* W303dGP0, which carries the P0 gene under the control of the inducible GAL1 promoter (*25*, *10*). When yeasts are growing in glucose medium, only the gene in the plasmid is expressed, and consequently the effect of the mutations can be estimated. The growth of strains transformed with either mutated gene is similar to that of the control strain transformed with the wild-type gene in galactose and glucose in either standard solid or liquid medium at 30 °C (Figure 2). In addition, the sensitivity of the transformed strains to salt and temperature was tested by growing them in either minimal YNB medium or in rich YEP medium in the presence of 0, 0.3, and 0.8 M NaCl at 20, 30, and 37 °C. Under no conditions was any difference detected in the growth rate of the strains expressing either the mutated or the wild-type gene (data not shown).

Phosphorylation of the Mutated Proteins. Phosphorylation of ribosomes was carried out in vitro as well as in vivo to test the modification of the mutated proteins as indicated in Materials and Methods. In the first case, the particles obtained from cells expressing the different mutated proteins and the controls were incubated with [*γ*-^{32}P]ATP and the ribosomal proteins were resolved by SDS−polyacrylamide gel electrophoresis. In the second instance, cells were grown in glucose medium in the presence of $^{32}PO_4^{3-}$, and the

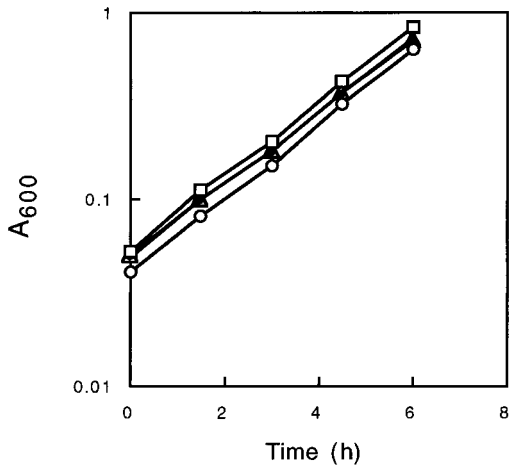

FIGURE 2: Growth of *S. cerevisiae* W303dGP0 transformed with plasmids pFL37-P0 (□), pFL37-P0C (●), pFL37-P0T (△), and pFL37-P0D (○) in YEP−glucose medium at 30 °C.

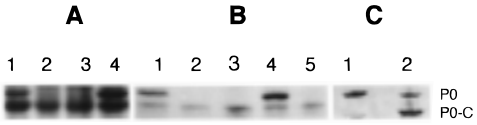

FIGURE 3: Phosphorylation of protein P0. (A) *S. cerevisiae* ribosomes (30 μg) containing either wild-type P0 (lane 1) or mutated P0 with cysteine (lane 2), aspartic acid (lane 3) or threonine (lane 4) in position 302, were labeled with $^{32}PO_4^{3-}$ in vitro, as indicated in Materials and Methods, and resolved by electrophoresis in 10% acrylamide gels. The labeled bands were detected by autoradiography. (B) Ribosomes from cells labeled in vivo were resolved as in panel A. Lanes 1−4 are as in panel A. Lane 5 correspond to ribosomes from *S. cerevisiae* W303dGP0 expressing a P0 protein lacking the last 21 amino acids from the C-terminal domain (*10*). (C) Ribosomes equivalent to those in lanes 1 and 5 in panel B were resolved similarly, and the P0 bands were detected by western blotting with a yeast P0-specific rabbit antibody. The positions of the wild-type and truncated proteins are indicated.

ribosomes were prepared and similarly analyzed by electrophoresis. The band corresponding to protein P0 was identified in the gels by using specific antibodies and microsequencing after blotting to PVDF membranes. The experimentally obtained sequence was XGIREKKA, which corresponds to the expected P0 amino-terminal sequence and indicates, in addition, that the P0 amino acid sequence has a free amino end.

In both labeling conditions, the autoradiogram of the respective gels indicated that the band corresponding to P0 is not labeled in the case of the proteins carrying cysteine and aspartic acid in position 302 (Figure 3). On the other hand, the protein in which the serine was replaced by a threonine seems to be more susceptible to phosphorylation and shows substantially higher labeling than the wild-type protein in vivo as well as in vitro.

In the in vivo experiment, ribosomes from *S. cerevisiae* W303dGP0-pC were also included. As previously described (*10*), this strain was obtained by transforming *S. cerevisiae* W303dGP0 with a plasmid carrying a truncated P0 gene lacking the C-terminal region encoding the last 21 amino acids. Labeling of protein P0 is not detected in this sample (lane 5, Figure 3B) supporting the notion that serine 302 is the site of P0 phosphorylation. The position of the truncated P0 protein is indicated in Figure 3C, which shows a western test of ribosomes from *S. cerevisiae* W303dGP0-pC grown

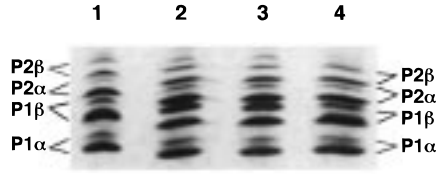

FIGURE 4: Isoelectrofocusing in a 2.5−5.0 pH range of ribosomes (1 mg) from *S. cerevisiae* W303dGP0 expressing either wild-type P0 (lane 1) or mutated P0 with cysteine (lane 2), aspartic acid (lane 3) or threonine (lane 4). The upper and lower bands in each protein correspond to the dephosphorylated and phosphorylated forms of the protein, respectively.

in glucose for 2 h. In these conditions some intact P0 can still be detected from the remaining galactose-grown ribosomes.

Effect of P0 Mutation on the Binding of the 12 kDa Acidic Proteins. To test whether the P0 mutation affects the composition of the stalk, ribosomes from the cells expressing the three mutated proteins, along with the wild-type polypeptide as a control, were analyzed by isoelectrofocusing in a 2.5−5.0 pH range (Figure 4). In these conditions the four acidic ribosomal proteins can be resolved. Some nonreproducible differences can be detected between the phosphorylated and dephosphorylated forms of some proteins, which are probably due to phosphatase activity during ribosome preparation. Considering both protein forms as a whole, no remarkable alteration can be perceived in the protein patterns from the four samples, indicating that P0 phosphorylation does not seem to notably affect its interaction with the P1- and P2-type proteins to form the ribosomal stalk.

Expression of P0 Mutated Proteins in a 12 kDa Acidic Protein-Defective Strain. Since the protein complex forming the ribosomal stalk is composed of five proteins having highly similar carboxyl ends, the lack of effect of the P0 mutations on the ribosome activity could be due to a compensatory action of the remaining components, the P1- and P2-type acidic proteins. To test this possibility, the mutated genes were expressed in *S. cerevisiae* D67dGP0. Like *S. cerevisiae* W303dGP0, the genomic P0 gene is in this strain under the control of GAL1, and, in addition, the *rpP1*α and *rpP1*β genes have been inactivated by disruption. Obviously, the ribosomes from D67dGP0 do not have P1 proteins, but the P2 proteins are not detected in the particle either, and their stalk only contains protein P0 (*26*).

S. cerevisiae D67dGP0 was transformed with each one of the plasmids pFL37-P0C, pFL37-P0T, pFL37-P0D, and pFL37-P0. All the His^+ transformant clones isolated, expressing either the mutated or the wild-type genes, grew with a similar growth rate in rich medium at 30 °C (Figure 5). Nevertheless, some phenotypic effect could be detected when cells were grown in nonstandard conditions. Thus *S. cerevisiae* D67, carrying a wild-type P0, is unable to grow on plates at 37 °C, but this temperature sensitivity is overcome by growing the cells in the presence of either 0.3 M NaCl (Figure 6) or 0.8 M sorbitol (data not shown). Growth in these conditions is not affected by the expression of either the aspartic acid or the cysteine P0 mutations but does not take place in the case of substitution by threonine.

In Vitro Activity of the Mutated Ribosomes. To test whether the effect of the threonine mutation was due to a temperature sensitive character of the mutated P0 protein that would inactivate the polypeptide at 37 °C, thus inhibiting

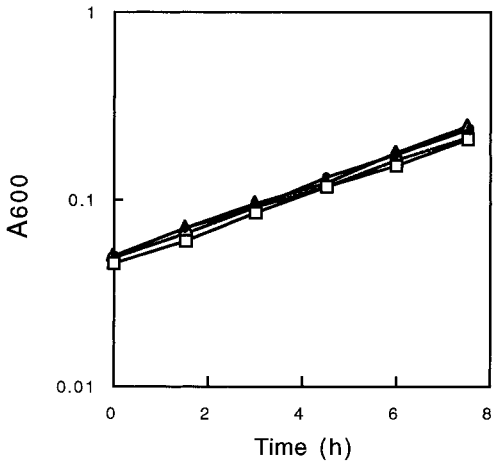

FIGURE 5: Growth of *S. cerevisiae* D67dGP0 transformed with plasmids pFL37-P0 (□), pFL37-P0C (●), pFL37-P0T (△), and pFL37-P0D (○) in YEP-glucose medium at 30 °C.

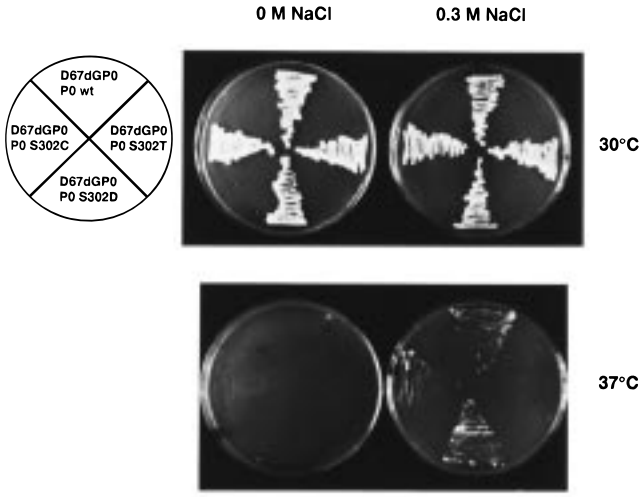

FIGURE 6: Growth of *S. cerevisiae* D67dGP0 transformed with plasmids pFL37-P0, pFL37-P0C, pFL37-P0T, and pFL37-P0D in YEP−glucose medium plates at 30 and 37 °C in the absence and in the presence of 0.3 M NaCl as indicated.

cell growth, an in vitro protein poly(U)-dependent synthesizing assay was performed. Ribosomes from *S. cerevisiae* D67dGP0 transformed with either pFL37-P0T or pFL37-P0 were obtained and tested in a poly(U)-dependent polymerizing system. The tests were performed at 30 and 37 °C using a supernatant fraction from either wild-type (W303) or mutant D4567 cells. *S. cerevisiae* D4567 is totally deprived of 12 kDa acidic proteins (*11*), and therefore a possible effect of these proteins, present in the wild-type supernatant, on the response of the D67 ribosomes to the temperature can be excluded by using a D4567-derived S100 fraction. Ribosomes from W303 cells were also used as a control (Table 2). In all cases a reduction in the activity of the polymerizing system was found at 37 °C, although the D67 seem to be more sensitive to the temperature than the wild-type particles, however, the decrease in activity was similar in all the D67 samples, independently of the P0 protein expressed.

DISCUSSION

Replacement of serine 302 in the amino acid sequence of the *S. cerevisiae* protein P0 abolishes its in vivo and in vitro

Table 2: Polymerizing Activity of Ribosomes at Different Temperatures[a]

S. cerevisiae strain		phenylalanine polymerized at		
ribosomes	S-100	30 °C (pmol)	37 °C (pmol)	% reduction
W303	W303	21.5	14.8	31.9
D67−302wt	W303	16.7	7.4	55.7
D67−302T	W303	15.1	6.3	58.3
D67−302wt	D4567	10.0	4.4	56.0
D67−302T	D4567	9.9	4.5	54.5

[a] Tests were performed as described in Materials and Methods with ribosomes and S-100 supernatant fraction from the indicated yeast strain. The average of three experiments is shown. Blanks in the absence of ribosomes, which ranged from 0.2 to 0.4 pmol, were subtracted in all cases.

phosphorylation, indicating that this residue must be the site of modification of the ribosomal component. Serine 302 is part of a conventional CKII phosphorylation site, SDDD, included in the carboxyl end of the protein. An identical C-terminal peptide is present in the other components of the ribosomal stalk—the yeast 12 kDa proteins P1 and P2—which have also been reported to be phosphorylated at an equivalent position (*12*−*14*). Conversely, *Tetrahymena* P proteins, which are the only eukaryotic polypeptides of this type that have not been found phosphorylated in the ribosome (*27*), lack a serine in the equivalent position (*28*).

On the other hand, the introduction of a threonine at position 302 of P0 notably increases the protein level of phosphorylation. This results is unexpected since, although threonine can also be modified by CKII, the replacement of a serine by a threonine in CKII substrates notably reduces the K_m and V_{max} of the phosphorylation reaction (*29*). The P proteins can be in vitro substrates for CKII (*13*, *30*, *31*); however, a number of other protein kinases have also been reported to phophorylate these ribosomal components (*32*−*34*). No data about the enzyme involved in the in vivo P protein phosphorylation have been reported, but the unexpected higher modification of the threonine containing protein P0 could indicate that CKII is not the enzyme involved in the process.

The C-end domain is highly conserved in the stalk proteins from all eukaryotic species (*8*). Since these proteins interact with the ribosome by their N-terminal domains (*35*), the carboxyl end is assumed to be located at the tip of this ribosomal protuberance and to be involved in its interaction with the supernatant factors, like in bacteria (*36*). A direct visual confirmation of this interaction was recently reported in bacteria by electron microscopy (*37*, *38*). Therefore, it would be not surprising that the presence of a phosphate residue can affect the function of the domain.

It was previously reported that dephosphorylation of the P1 and P2 proteins inactivates the capacity of the proteins to reconstitute active ribosomes in vitro (*15*, *16*), and recently, results from in vitro experiments have suggested that protein P2 phosphorylation is involved in the interaction with the elongation factor EF-2 (*17*). This conclusion has not, however, been confirmed in vivo. Thus, mutation of the phosphorylatable serine residue in individual *S. cerevisiae* P1 and P2 proteins did not affect the functionality of the ribosome, since the mutant strains have the same growth rate as the controls in standard conditions (*14*). However, the

possibility could not be totally excluded that the defect caused by one mutant protein could be overcome by the other wild-type components on the five-protein stalk complex (*3*).

The results in this report indicate that mutations in serine 302, which prevent P0 modification, do not substantially affect cell growth. *S. cerevisiae* W303dGP0, a conditional null mutant carrying the P0 gene under the control of the GAL1 promoter and consequently unable to grow in glucose, grows in this sugar with an identical doubling time when transformed with a plasmid containing either the P0 wild-type gene or a mutated gene encoding an unphosphorylatable protein under the control of a constitutive promoter. In addition, the composition of the stalk is not notably affected by the presence of the unmodified P0. It seems, therefore, that phosphorylation of P0 is unnecessary for protein synthesis in standard conditions and in the presence of the remaining phosphorylated stalk components. Moreover, P0 modification is not a requirement for the binding of the 12 kDa acidic proteins to the ribosome either.

The same results are obtained when the mutated proteins are expressed in *S. cerevisiae* D67. In this strain, the genes encoding the 12 kDa proteins P1α and P1β have been inactivated and, in the absence of these components, proteins P2α and P2β cannot bind to the ribosome (*26*). The ribosomes obtained from strain D67 are totally deprived of P1 and P2 proteins, and consequently, in this strain protein P0 alone must carry out the functions played by the whole stalk in the wild-type ribosome. These P1/P2-deprived ribosomes translate mRNA at a lower rate but with an accuracy similar to that of the control particles (*11*). Therefore, the fact that the expression of the mutated proteins does not affect the growth rate of *S. cerevisiae* D67 confirms that P0 phosphorylation is not essential for the overall translation process even in the absence of the remaining stalk components at 30 °C in standard medium.

However, some phenotypic effects of the mutation at serine 302 in *S. cerevisiae* D67 are detected at 37 °C in 0.3 M NaCl. This strain is unable to grow at 37 °C, but growth takes place if an osmotic stabilizer, NaCl or sorbitol, is included in the medium. In these conditions, the P0 protein carrying a threonine at position 302 is unable to support growth. This effect is not due to inactivation of the mutated protein at high temperature, since an in vitro polymerizing system derived from the strain expressing the altered protein is affected similarly to the controls at 37 °C. As commented previously, this mutation substantially increases the level of P0 phosphorylation. At the moment, it is not possible to determine whether this phenotype is due to this effect on the protein modification or is a consequence of the conformational changes introduced by threonine, although the lack of effect in the case of the aspartic acid and cysteine mutations seems to point to the first explanation. In this sense, it must be noted that a mutation affecting the YP1α, protein phosphorylation site has also been shown to have a phenotype related to osmotic and temperature sensitivity when expressed in *S. cerevisiae* D67 (14).

Considering that changes in the stalk structure affect differentially the translation of various mRNAs, resulting in an altered pattern of expressed proteins (*11*), it is possible that the level of the stalk phosphorylation can also preferentially affect the expression of some proteins. Changes in the phosphorylation of the stalk components, P0, P1, and P2, could affect the translation of specific mRNAs differentially, altering in this way the relative concentration of the different components of the metabolic pathways. These alterations could result in important phenotypic effects. Our results seem to indicate that the osmoregulation of the cell is especially sensitive to the changes in the stalk phosphorylation. Complementary experimental evidence is required to confirm this hypothesis. It seems clear, in any case, that although phosphorylation of P0, and probably of the whole stalk, does not have an important effect on the overall translating activity of the ribosome, it is able to elicit changes in this ribosomal active domain that can have important phenotypic consequences in some specific conditions.

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