In vivo $^{32}$P-labeled yeast proteins from wild type and ppz1 ppz2 phosphatase mutants were resolved by bidimensional electrophoresis. A prominent phosphoprotein, which in ppz mutants showed a marked shift to acidic regions, was identified by mixed peptide sequencing as the translation elongation factor 1Bα (formerly eEF1β). An equivalent shift was detected in cells overexpressing HAL3, a inhibitory regulatory subunit of Ppz1. Subsequent analysis identified the conserved Ser-86 as the in vivo phosphorylatable residue and showed that its phosphorylation was increased in ppz cells. Pull-down experiments using a glutathione Stransferase (GST)-EF1Bα fusion version allowed to identify Ppz1 as an in vivo interacting protein. Cells lacking Ppz display a higher tolerance to known translation inhibitors, such as hygromycin and paromomycin, and enhanced readthrough at all three nonsense codons, suggesting that translational fidelity might be affected. Overexpression of a GST-EF1Bα fusion counteracted the growth defect associated to high levels of Ppz1 and this effect was essentially lost when the phosphorylatable Ser-86 is replaced by Ala. Therefore, the Ppz phosphatases appear to regulate the phosphorylation state of EF1Bα in yeast, and this may result in modification of the translational accuracy.

The elongation step of protein synthesis involves the binding of aminocyl-tRNA to the ribosomal “A” site, formation of a peptide bond, and translocation of the newly formed peptidyl-tRNA to the “P” site. The elongation factor 1 (EF1) is responsible for the GTP-dependent binding of aminocyl-tRNA to the ribosomal A site in polypeptide chain elongation and participates in proofreading of the codon-anticodon match (1).

In the budding yeast Saccharomyces cerevisiae, EF1 consists of different subunits: EF1A (formerly EF1α) is encoded by two different genes (TEF1 and TEF2), and it binds aminocyl-tRNA in a GTP-dependent manner. The exchange of GDP for GTP on EF1A is stimulated by a member of the guanine nucleotide exchange factor family, EF1Bα, which is encoded by a single gene (TEF5). An additional subunit of uncertain function is encoded by genes TEF3 and TEF4. Although lack of TEF3 and TEF4 results in no observable defects in translation (2), lack of TEF5 or simultaneous deletion of TEF1 and TEF2 is lethal (3, 4). Components of EF1 have been shown to be phosphorylated in vitro by diverse protein kinases in species different from yeast (5–7).

The function of EF1Bα on EF1A has been shown to be critical for an efficient and accurate translation. For example, cells with increased expression of the EF1A subunit can bypass the lethality of cells lacking EF1Bα. However, these cells present a number of defects, including higher sensitivity to inhibitors of translation elongation and changes in translational fidelity (4). Alterations in translational fidelity have also been produced by specific mutations in EF1Bα, as it has been documented by evaluation of sensitivity to drugs such as paromomycin and analysis of translational fidelity at nonsense codons (8). The fidelity of translation may be related, at least in part, to the requirement for nucleotide exchange, as it has been tested by mutations in the GTP-binding motif of yeast EF1A (9).

The yeast Ppz phosphatases are encoded by genes PPZ1 and PPZ2 (10, 11, 12) and represent a novel type of Ser/Thr phosphatases characterized by a catalytic carboxyl-terminal half related to type 1 phosphatase. These phosphatases are involved in a variety of cell processes, including maintenance of cell integrity, in connection with the Pkc1/Mpk1 mitogen-activated protein kinase pathway (11, 12), regulation of salt tolerance (13), and regulation of cell cycle at the G1/S transition (14). In all cases, the function of Ppz1 appears to be more important than that of Ppz2. Recently, we have identified the halotolerant determinant Hal3 as a negative regulatory subunit of Ppz1 that modulates the diverse physiological functions of the phosphatase (15).

As an attempt to better understand the physiological role of the Ppz phosphatases, we have performed a two-dimensional electrophoretic analysis of proteins from in vivo $^{32}$P-labeled wild type and ppz strains, in search for polypeptides that might display an altered phosphorylation state in the absence of the phosphatases. This approach has led us to establish a previously unsuspected link between the Ppz phosphatases and the translation elongation factor 1Bα.
**Experimental Procedures**

**Strains and Growth Conditions**—*Escherichia coli* strains NM522 or DH5*α* were used as a host for DNA cloning. Bacterial cells were grown at 37 °C in LB medium containing 50 μg/ml ampicillin, when needed, for plasmid selection. Yeast cells were grown at 28 °C in YPD medium or, when indicated, in synthetic minimal (SD) or complete minimal (CM) medium (16). Most yeast strains used in this work derive from JA-100 (MATa PRZ2 PRZ2 ura3-52 leu2-3,112 trp1-1 his4 can1-1). Construction of JA-101 (MATα prz1:URA3), JA-103 (MATa prz2::TRP1), and JA-105 (MATα prz1:URA3 prz2::TRP1) has been described previously (14). Strain EDN75 (MATα prz1::Kan) was made by replacing the entire PRZ1 ORF with the KanMX4 module (17), using oligonucleotides PRZ1/SFH1 5′-CCA TTC TCT GCT TAT TTT TCC TTC TCT AAA AGG CCC AGT CAG-3′ and EFb3′ 5′-CGG ATC TTT TTA ATG TTT GGAT CAT GAC-3′. The underlined residues denote the engineered BamH1 site used in plasmid ORF amplification in frame with the GST sequence of plasmid p426TEG2, to yield p426TEF5. Plasmid p426TEG2 is based on plasmid pRS/TEF5. Plasmid DNA was recovered and sequenced to identify clones with the correct orientation and lacking unwanted mutations.

To identify proteins of interest, membranes were stained with Amido Black and subjected to autoradiography. Protein staining and phospho-tyrosine patterns were compared using Melanie software (Bio-Rad). Relevant fragments were sliced, removed, digested for 90 min with 200 μl of 500 mg/ml cyanogen bromide, as described (23), and subjected to amino acid sequencing in a Applied Biosystems Procise 494 apparatus. The mixed sequenced segments were run against the FASTF data base to select the protein that matched.

To identify the nature of the phosphorylated residue(s), strain EDN75 (ppz1::KAN) was transformed with plasmid p426TEF5, and cells were labeled with 32P as above, recovered by centrifugation, and disrupted with glass beads in 25 ml Tris-HCl buffer (pH 7.5), containing 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 0.5 mM PMSF, 0.5 mM benzamidine, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The homogenate was centrifuged at 100,000 × g for 60 min at 4 °C, and the supernatant was loaded into a 1-ml glutathione-Sepharose column equilibrated with the above mentioned buffer. The column was washed with the same buffer plus 250 mM NaCl, and the fusion protein was eluted with 10 mM glutathione. The purified GST-EF1β was the only radioactive band when analyzed by SDS-PAGE. The protein (0.5 mg) was digested with endolysyl peptide C (20 μg/ml), which cuts carboxyl-terminal to lysine residues. Digests were acidified with TFA, and applied to a reverse phase column (Waters Nova-Pak C18, 3.9 × 150 mm) that had been equilibrated in 0.1% TFA (buffer A). The flow rate was maintained at 1 ml/min. The column was washed for 10 min with buffer A before peptides were eluted with a linear gradient of acetonitrile (0–80% in 80 min) in buffer B. Fractions (1 ml) were collected, and peptides containing 32P were identified by measuring Cerenkov radiation. Those fractions were pooled and evaporated to dryness before the peptides were immobilized to Immobilon membranes (Millipore) following the manufacturer’s instructions (28). The peptide identified from in vivo labeled EF1β were identified with a vapor phase amino acid sequencer (Applied Biosystems Procise 494). Phosphorylated residues within phosphopeptides were located by determining the cycles in which 32P was released when samples were subjected to sequential Edman degradation under conditions that optimize recovery of 32P (25).

**Immunodetection of Ppz1 Bound to Purified GST-EF1β Fusion Protein**—Wild type strain JA-100 and the ppz1 mutant strain EDN75 were transformed with the empty plasmid p426TEG2 and plasmid p426TEF5. Cells were grown in synthetic medium lacking uracil up to an OD600 of 2, recovered by centrifugation, and disrupted with the aid of glass beads in 50 mM Tris-HCl buffer (pH 8.0), containing 1 mM EDTA, 2 mM dithiothreitol, 150 mM NaCl, 0.5 mM PMSF, 0.5 mM benzamidine, 1 μg/ml leupeptin and 1 μg/ml pepstatin. A crude extract was prepared by centrifugation at 50,000 × g for 1 min, and 1 mg of protein was incubated with 100 μl of glutathione-Sepharose beads for 90 min at 4 °C. Beads were washed with the above mentioned buffer, resuspended in SDS-sample buffer, and boiled, and the supernatant was loaded in a 8% SDS-polyacrylamide gel. After transfer to Immobilon-P membranes (Millipore), the presence of Ppz1 was assessed by immunoblotting against anti-Ppz1 antibodies as described previously (15).

**Nonsense Suppression Assays**—Suppression of nonsense codons was estimated by using the pUKC series plasmids (26). Plasmid pUKC815 carries the lacZ gene under the LACZ and PGK promoter. Plasmids pUKC817, pUKC818, and pUKC819 are identical to pUKC815, but they carry the nonsense codons UAA, UAG, and UGA at the beginning of the β-galactosidase coding sequence. Yeast strains were transformed with these plasmids and grown up to an OD600 of 0.5–1.0 in SD medium lacking uracil, and the β-galactosidase activity was determined as described previously (27).

**Sensitivity to Drugs and Growth Assays**—For testing the sensitivity to various drugs on plates, cultures were grown until an OD600 of 2 and 100 μl of the culture was spread on YPD plates. 20 μl of a solution of each drug was placed on sterile disks on top of the plates, and growth resumed at 30 °C for 2–3 days. Sensitivity in liquid medium was assessed as follows. Exponential cultures were diluted up to an OD600 of 0.01. Aliquots of 200 μl were supplemented with 100 μl of medium containing the appropriate amount of the drug. Growth was resumed for 16–18 h, and sensitivity to drugs was monitored by measuring the OD600 of the culture.
The absence of Ppz phosphatase activity affects the phosphorylation state of EF1Bα. Wild type strain JA-100 (WT), strain JA-105 (ppz1 ppz2), or wild type cells overexpressing the gene encoding the negative regulatory subunit HAL3 (HAL3) were 32P-labeled, and phosphopeptides were analyzed by two-dimensional gel electrophoresis. IEF indicates isoelectric focusing, and SDS denotes the second dimension electrophoresis (SDS-PAGE). Silver staining of the gels is shown in the upper panel, whereas the lower panel displays the corresponding autoradiograms.

RESULTS

Yeast EF1Bα Is a Phosphoprotein in Vivo Whose Phosphorylation State Is Affected by Ppz Phosphatases—To evaluate the influence of lack of Ppz phosphatases in the cellular phosphorylation pattern, wild type and ppz1 ppz2 yeast cells were 32P-labeled and total cell extracts prepared and subjected to bidimensional electrophoresis. Global analysis of the distribution of phosphopeptides did not show a remarkable overall modification of the pattern. However, a clear shift to more acidic regions of a phosphoprotein of about 22.5 kDa and focusing at pH 4.3 was observed in the double mutant ppz1 ppz2 compared with the wild type yeast cells (Fig. 1). The acidic shift of this protein was also observed when the gel was silver-stained, indicating that it was a relatively abundant component. Because shifting to more acidic regions is often associated to increased phosphate content, this result was considered as indicating that this protein could be a target for the Ppz phosphatases. To further test this possibility, a similar experiment was carried out using wild type cells that contained a high copy plasmid carrying the HAL3 gene, which codes for a negative regulator of Ppz1 (15). As shown in Fig. 1, overexpression of HAL3 resulted in a pattern identical to the one observed in ppz mutants, supporting the notion that the shift was the result of lack of Ppz activity.

The identity of the mentioned phosphoprotein was established by recovering the region of the membrane, followed by digestion with cyanogen bromide and automated Edman sequencing of the resulting peptide mixture. Analysis of yeast protein data banks indicated that this protein corresponded to the product of the single-copy, essential TEF5 gene, encoding the eukaryotic elongation factor 1Bα (3). Therefore, yeast EF1Bα is a phosphoprotein in vivo, and its phosphorylation state may be modified by the Ppz phosphatases.

To identify the residue(s) phosphorylated in vivo, a GST-EF1Bα fusion protein was expressed from plasmid p426/TEF5 in 32P-labeled yeast cells. The product was affinity purified by glutathione-Sepharose chromatography and digested with endolysyl peptidease C. Phosphopeptide mapping by HPLC analysis indicated that all in vivo phosphorylation sites are located in a single endolysyl peptide C fragment (Fig. 2). Phosphoamino acid analysis of both the entire labeled fusion protein or the relevant chromatographic fractions revealed that all radioactivity was bound to Ser residue(s) (Fig. 2). Further analysis of the radioactive endolysyl peptide C fragment by measuring recovery of 32P during Edman sequencing proved that radioactivity was associated to a single residue that was determined to be Ser-86.

Affinity-purified GST-EF1Bα from Yeast Cells Contains Bound Ppz1—The possibility that the Ppz phosphatases might interact in vivo with the elongation factor was approached by using an affinity system based on the expression of EF1Bα in yeast as a GST fusion protein. This recombinant protein was affinity-purified, and the presence of accompanying proteins was evaluated by SDS-PAGE. When the presence of Ppz1 was tested by immunoblot in these samples (Fig. 3), it was found that affinity-purified EF1Bα contained bound Ppz1. This finding indicates that Ppz1 and EF1Bα can interact in vivo and leads to the possibility that Ppz1 could directly dephosphorylate EF1Bα. In fact, when the GST-EF1Bα fusion protein was expressed in 32P-labeled cells from the low copy plasmid pRS/TEF5, an increase in radioactive phosphate content of about 2-fold was observed in ppz cells when compared with the wild type strain (data not shown). However, our attempts to in vitro dephosphorylate the in vivo 32P-labeled translation factor using available bacterially expressed Ppz1 have been so far unsuccessful.

Evidence for Functional Interactions between Ppz Phosphatases and EF1Bα—The observation that the Ppz phosphatases may affect the in vivo phosphorylation state of EF1Bα prompted us to analyze phenotypes related to changes in the function of this protein. It has been recently reported that mutations in the conserved carboxyl terminus of EF1Bα alter the sensitivity of yeast cells to translation elongation inhibitors. We tested the sensitivity of wild type and phosphatase-deficient strains to paromomycin, hygromycin B, and cycloheximide by both liquid cultures and the halo assay. As shown in Fig. 4, deletion of both phosphatase genes clearly increased the sensitivity of yeast cells when compared with the wild type strain (data not shown). However, our attempts to in vitro dephosphorylate the in vivo 32P-labeled translation factor using available bacterially expressed Ppz1 have been so far unsuccessful.

Changes in sensitivity to paromomycin have been related to altered translational fidelity. Therefore, we sought to investigate whether the absence of Ppz phosphatase activity might affect this cellular function, by evaluating the suppressor capacity of the ppz mutant strains. To this end, we transformed to...
Regulation of eEF1Bα by Phosphatases

The fusion protein GST-EF1Bα was expressed in wild type (PPZ1+) or EDN75 cells (PPZ1−) and affinity-purified from 1 mg of total yeast extracts through a glutathione-Sepharose matrix. An additional control in which wild type cells were transformed with the empty p426 plasmid was also included. After extensive washing the samples were resuspended in sample buffer, electrophoresed in SDS-polyacrylamide gels, and transferred to membranes. The presence of interacting Ppz1 was tested by immunoblot using available anti-Ppz1 antibodies developed against recombinant GST-Ppz1 protein. Note that these antibodies also recognize the GST moiety of GST-EF1Bα.

![Figure 3. Immunodetection of Ppz1 bound to yeast GST-EF1Bα.](image)

**FIG. 3.** Immunodetection of Ppz1 bound to yeast GST-EF1Bα. The fusion protein GST-EF1Bα was expressed in wild type (PPZ1+) or EDN75 cells (PPZ1−) and affinity-purified from 1 mg of total yeast extracts through a glutathione-Sepharose matrix. An additional control in which wild type cells were transformed with the empty p426 plasmid was also included. After extensive washing the samples were resuspended in sample buffer, electrophoresed in SDS-polyacrylamide gels, and transferred to membranes. The presence of interacting Ppz1 was tested by immunoblot using available anti-Ppz1 antibodies developed against recombinant GST-Ppz1 protein. Note that these antibodies also recognize the GST moiety of GST-EF1Bα.

![Figure 4. ppz1 mutants are hypertolerant to inhibitors of translational fidelity.](image)

**FIG. 4.** ppz1 mutants are hypertolerant to inhibitors of translational fidelity. Upper panel, YPD medium containing the indicated concentrations of drug was inoculated (initial OD660 of 0.007) with wild type JA-100 (○), JA-101 (ppz1, □), or JA-105 (ppz1 ppz2, ▽) cells. Cultures were grown for 18 h, and the density of the cultures was then measured. Relative growth was calculated as the ratio between growth in the presence or the absence of added drug and expressed as a percentage. Data are mean ± S.E. from four independent experiments performed by triplicate. Lower panel, lawns of JA-100 (WT), JA-101 (ppz1), or JA-105 (ppz1 ppz2) were prepared on plates with sterile filters containing 30 μM hygromycin B (Hyg) or 0.025 mM cycloheximide (Cyclo). Growth was monitored after 3 days.

![Figure 5. Nonsense suppressor capacity of Ppz1-deficient yeast cells.](image)

**FIG. 5.** Nonsense suppressor capacity of Ppz1-deficient yeast cells. Wild type JA-100 (filled bars) and Ppz1-deficient EDN75 (dashed bars) strains were transformed with plasmids pUKS17 (p17), pUKS18 (p18), and pUKS19 (p19) that carry the nonsense codons UAA, UAG, and UGA, respectively, at the beginning of the β-galactosidase coding sequence. Cells were grown up to an OD660 of 0.5–1, and β-galactosidase activity was determined using the chromogenic substrate o-nitrophenyl-β-D-galactoside. Data are mean ± S.E. from four independent clones determined by triplicate.

![Table](table)

**DISCUSSION**

In this report we demonstrate that, in the yeast *S. cerevisiae*, translation elongation factor 1Bα is a phosphoprotein. Phosphorylation site mapping and sequence analysis indicates that the Ser-86 is the only phosphorylatable residue in this protein, at least under standard growth conditions. It is remarkable that data base search reveals that the equivalent Ser residue (as well as its acidic environment) is also found in a large variety of organisms, including *Drosophila melanogaster, Cae-

norhabditis elegans*, mouse, and human. Phosphorylation of EF1Bα has been reported in *Artemia salina* (5), wheat (6), and reticulocyte (7). In the former case, phosphorylation was ascribed to Ser-89, which is equivalent to Ser-86 in yeast EF1Bα. Interestingly, phosphorylation has been correlated to changes in its catalytic nucleotide exchange activity, although reports are somewhat contradictory (5, 7).

Our data indicate that deletion of the *ppz* genes and overexpression of Hal3, a negative regulatory subunit of Ppz1 (15),
result in increased phosphorylation of the EF1Bα protein, specifically at Ser-86. These results would be compatible with a role of Ppz1 in regulating the phosphorylation state of the translation factor and, possibly, its function. We also show here evidence that affinity-purified yeast EF1Bα contains significant amounts of bound Ppz1, by using an approach that was pivotal in the past to identify the Hal3 protein as a subunit of Ppz1 (15). This could be taken as an indication that Ppz1 could be able to directly dephosphorylate EF1Bα. However, we have been unable to detect direct dephosphorylation of either in vivo labeled or CK-2 in vitro phosphorylated EF1Bα in the presence of bacterially expressed Ppz1. Although at this point we cannot provide direct evidence for the translation factor being a substrate for the phosphatase, this possibility formally remains. For instance, the phosphatase might require accessory proteins (absent in our in vitro assay) to effectively use EF1Bα as substrate. In this regard, there is a large body of evidence for the requirement of specific regulatory subunits (targeting subunits) for Ser/Thr phosphatases to localize at specific subcellular sites or to use a given phosphoprotein as an effective substrate (28, 29). It must be noted that dephosphorylation events have been previously related to the control of the accuracy of protein synthesis, as it is the case of the Ppq1/Sal6 Ser/Thr protein phosphatase (30, 31), the closest structural homologue of the Ppz phosphatases. However, the possible role of this phosphatase has not been worked out.

We considered that if EF1Bα was a target (either direct or indirect) for Ppz1, it could be possible to establish some sort of functional connection between both proteins. Deletion of TEF5 is lethal for the cell, and high copy expression of TEF2 suppresses the lethal phenotype of tef5 mutants (4). However, these cells are markedly sensitive to translational inhibitors, such as paromomycin and hygromycin B. It is remarkable that lack of Ppz phosphatases also results in a change in sensitivity to these compounds, although in this case yielding more tolerant cells. Because these drugs are aminoglycosides known to enter the yeast cell driven by the membrane potential, which is mostly maintained by the function of the membrane H⁺-ATPase (32), we considered the possibility that the increased tolerance could be an indirect effect due to altered proton efflux. However, this was ruled out by determining this parameter in wild type and ppz mutants and finding essentially identical values (data not shown).

Changes in sensitivity to paromomycin have been related to altered translational fidelity (33, 34), a phenotype also produced by changes in the dosage of EF1A (35). Recent evidence (8) has been presented pointing out that mutations in the carboxyl-terminal region of EF1Bα results in increased sensitivity to translation inhibitors and that this effect was accompanied by enhanced translational fidelity (i.e. reduced readthrough at nonsense codons). These observations are in keeping with our finding that cells lacking Ppz phosphatases, which are more tolerant to certain translation inhibitors, show an increased readthrough at nonsense codons, most likely due to a decrease in translational fidelity.

Further evidence for a functional interaction between EF1Bα and Ppz1 comes from the observation that overexpression of the translation factor strongly attenuates the growth defect, due to a delayed G1/S transition, of cells containing an excess of Ppz1 activity. Although we showed in the past that this defect correlates with a delay in G1/S cyclin mRNA expression (14), immunoblot analysis of the protein level of different cyclins reveals that, at least in the case of Clb5, further post-transcriptional alterations (i.e. at the translation level) could exist.2 Remarkably, a non-phosphorylatable version of EF1Bα was unable to counteract the effect of an excess of Ppz1, suggesting that in vivo modulation of the phosphorylation state of the factor is somehow involved in the regulation of its function. It has been reported that, when expressed from the powerful GAL promoter, a carboxyl-terminal fragment of EF1Bα, lacking Ser-86, was sufficient for normal growth and did not display dramatically altered drug or temperature sensitivity (8). Furthermore, a strain containing a S86A version of EF1Bα as the only source for the factor is viable.3 Therefore, it must be concluded that regulation of EF1Bα by phospho-dephosphorylation at Ser-86 (which, at least in part, would involve Ppz1) must affect the function of the translation factor in a subtle way. From our data, it can be hypothesized that changes in the phosphorylation state of EF1Bα would result in altered nucleotide exchange on EF1A. However, alternative mechanisms cannot be excluded, because it has been postulated that EF1Bα may have additional regulatory effects on EF1A (9). In any case, our data provides further support to the notion that phospho-dephosphorylation mechanisms are relevant for a proper regulation of protein synthesis.

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A Role for the Ppz Ser/Thr Protein Phosphatases in the Regulation of Translation
Elongation Factor 1B α
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