Deletion of the yeast Ser/Thr protein phosphatase PPZ1 results in increased tolerance to sodium and lithium. PPZ1 is also important for cell integrity, as ppz1Δ cells undergo lysis under caffeine stress and PPZ1 overexpression overrides the lytic defect of mutants in the protein kinase C/mitogen-activated protein (MAP) kinase pathway. The PPZ1 protein can be dissected in two halves. The COOH-terminal half is related to type 1 phosphatases, whereas the NH2-terminal half is unrelated to phosphatases and contains a consensus site for N-myristoylation. Several mutated versions of PPZ1 have been constructed and tested for complementation of ppz1Δ mutants. We show that PPZ1 can be myristoylated in vivo and that change of Gly-2 to Ala results in lack of myristoylation and loss of complementation of salt tolerance. Removal of the entire NH2-terminal half results in complete loss of function, although it does not abolish the phosphatase activity of the protein expressed in Escherichia coli. The deletion of a large region of the NH2-terminal half (residues 17–193) does not affect the ability to complement the salt tolerance phenotype but abolish complementation of caffeine sensitivity, whereas the opposite behavior is observed upon removal of residues from 241 to 318. Mutation of Arg-451 to Leu results in both complete loss of function and of phosphatase activity. These results indicate that the NH2-terminal half of the protein contains structural determinants that are specific for certain functions and that the phosphatase activity is required but not sufficient for full PPZ1 function.

In addition to the classical Ser/Thr protein phosphatases, namely types 1, 2A, 2B, and 2C (1–3), yeast cells contain a number of novel phosphatases. A most interesting example of these novel forms is the proteins encoded by genes PPZ1 (93%), whereas the NH2-terminal halves of PPZ1 and PPZ2 are more divergent from other phosphatase sequences (61%) and size (about 350 residues) with the catalytic subunit of type 1 phosphatase (PP-1) from different organisms, whereas the NH2-terminal region is completely unrelated to other phosphatase sequences and is characterized by a large number of Ser and Thr residues, as well as of basic amino acids. The NH2-terminal region of PPZ1 also contains a consensus sequence for N-myristoylation.

This is a remarkable feature since, in yeast, only a very limited number of proteins are known to be myristoylated in vivo (for review, see Ref. 5). Interestingly enough, another Ser/Thr protein phosphatase, calcineurin (protein phosphatase 2B) can also be myristoylated in vivo and this modification affects its regulatory subunit, encoded by the gene Cnb1 (6). Bacterially expressed PPZ1 has been characterized in our laboratory and found to display Ser/Thr protein phosphatase activity toward several substrates. Although in some aspects the activity of recombinant PPZ1 resembles that of PP-1, a number of differences (e.g. inability to dephosphorylate glycogen phosphorylase) were also observed (7). The gene PPZ2 (8, 9) codes for a 710-residue polypeptide that can also be considered as composed of two halves. The COOH-terminal one is very similar to the COOH-terminal half of PPZ1 (93%), whereas the NH2-terminal halves of PPZ1 and PPZ2 are more divergent from each other (43% of identity). Despite this higher divergence, the NH2-terminal half of PPZ2 is also rich in Ser and Thr residues and, as in PPZ1, contains a consensus sequence for N-myristoylation.

Strains lacking both PPZ1 and PPZ2 are prone to cell lysis under certain stress situations, such as exposure to caffeine (10) or high temperature (9) and the lytic phenotype can be rescued by addition of 1 M sorbitol to the medium as osmotic cushion. The contribution of each gene product to this phenotype is different, the lack of PPZ1 being responsible for more dramatic changes. The lytic phenotype is also found in mutants in the protein kinase C/MAP kinase pathway (9), involved in the proper construction of the yeast cell wall, and there is evidence that the PPZ phosphatases are somehow related to this pathway, since the deletion of PPZ1/PPZ2 is additive to the deletion of the MAP kinase gene, and this can be complemented by high copy number expression of both phosphatase genes (9).

Recently, our laboratory demonstrated that the PPZ phosphatases are also involved in salt homeostasis (11). Lack of PPZ1 (but not of PPZ2) results in increased tolerance to sodium and lithium cations. An additional increase is observed when PPZ2 is disrupted in a ppz1Δ background. These mutants display an increased output of sodium and lithium, as a result of an increase in the mRNA levels of the ENA1/PMR2 gene (11), encoding a P-type ATPase that is believed to be the major pump responsible for sodium (and lithium) efflux in yeast cells.
It is remarkable that calcineurin is also involved in salt homeostasis in yeast. In this case, however, lack of calcineurin results in increased salt sensitivity (15–17). Our findings indicate that the mechanisms of action of the PPZ phosphatases and calcineurin are independent and opposite (11).

The discovery of relevant phenotypic changes associated with the absence of the PPZ phosphatases (particularly of PPZ1) and the unusual nature of the NH2-terminal half of these proteins moved us to initiate this study, aiming to gain further insight into the structure-function relationship of these phosphatases.

**MATERIALS AND METHODS**

Growth of *Escherichia coli* and Yeast Strains—*E. coli* strains (strain NM522) were grown at 37 °C (unless otherwise stated) 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 SD synthetic medium (18). Yeast strains used in this work were DL790-3A (*MAT a can-1 ppz1::URA3 ppz2::TRP1*), previously linearized by ScaI (20). DNA sequencing was performed using the dideoxynucleotide chain termination method (21). Genomic yeast DNA was prepared after disruption of yeast cells with glass beads as described (21). Genomic yeast DNA was prepared after disruption of yeast cells with glass beads as described (21).

**Recombinant DNA Techniques—** *E. coli* cells were transformed using standard calcium chloride treatment (20). Yeast cells were transformed by a modification of the lithium acetate method (22). Genomic yeast DNA was prepared after disruption of yeast cells with glass beads as described (18). Restriction reactions, DNA ligations and other standard recombinant DNA techniques were performed essentially as described (20). DNA sequencing was performed using the dideoxynucleotide chain termination method (22) using an automated Applied Biosystems 373A DNA sequencer.

**Salt and Caffeine Sensitivity Assays—** The sensitivity of the cells to lithium chloride and caffeine was evaluated on freshly prepared SD agar plates containing different concentrations of the compound. Drop tests were performed essentially as in (11). Growth test in liquid cultures was performed as follows. Cultures were grown in SD medium for 2 days, and 30 μl of a 1:100 dilution was inoculated into 5 ml of YPD medium. Then, cultures were grown for 19 h in the absence of lithium chloride, and relative growth was determined as the value of the A600 in lithium-treated cultures in comparison with growth monitored in the same medium in the absence of salt.

**Construction of the Different Versions of PPZ1 for Low-copy Expression in Yeast—** To generate wild type and mutated versions of PPZ1 we devised an strategy based in the construction of a PPZ1 promoter cassette and its fusion to the different versions of the PPZ1 open reading frame. The promoter cassette was constructed as follows. A DNA fragment corresponding to nucleotides −800 to −10 (the A of the initiating Met being +1) was amplified from genomic DNA using oligonucleotides P4 and P2 (Table I), that contain *Bam*HI and *Sal*I sites, respectively. The source of DNA was not the previously cloned PPZ1 gene (4), because the initial clone did not contain the full promoter sequence.2 The amplification fragment, which has an internal *Bam*HI site located at about 0.33-kbp from the 5′ end, was digested with *Bam*HI and *Sal*I. This generated a 456-base pair DNA fragment displaying full promoter activity (not shown) that was cloned into the *Bam*HI and *Sal*I sites of plasmid YCplac111, which carries the LEU2 marker (23), yielding pYPBZ1. A wild type version of the PPZ1 open reading frame was generated by PCR using oligonucleotide C1 (which contains the initiating Met and a *Sal*I site at the 5′ end) and oligonucleotide C1 (which corresponds to the sequence from nucleotide +2256 to +2273, that is, about 175 nucleotides downstream the stop codon, and includes a * HindIII restriction site). The resulting 2.3-kbp fragment was digested with *Sal*I and *HindIII* and cloned into pYPBZ1, yielding pYCY1Z1.

An open reading frame lacking the entire NH2-terminal half was also generated by standard PCR techniques using oligonucleotides C2 and T1. C2 encodes from residues 345 to 349 of PPZ1, the first triplet being a Met codon, and has a *Sal*I site at its 5′ end. The resulting open reading frame codes for 348 residues and can be easily aligned with type 1 phosphatases from different origins (4). The 1.25-kbp amplification fragment was digested with *Sal*I and *HindIII* and cloned into pYPBZ1 to yield pYCZ2Z1.

Deletion of residues 241–318 of PPZ1 was accomplished by digestion of pYCZ12Z1 with *Nsi*I and religating the resulting 8.7-kbp DNA fragment to delete residues 175–193 was done as follows. The pYCZ12Z1 plasmid was digested with *Bam*HI and *HindIII* to liberate the entire insert, and this was cloned into pBluescript SK− (Stratagene). The construct was digested with EcoRI, and the resulting 5.3-kbp fragment was religated. The insert was recovered by digestion with *Bam*HI and *HindIII* and cloned back into YCplac111 to give pYCZ217Z1.

Change of the putative myristoylation site, Gly-2, to Ala was made as following: a PCR reaction was performed using oligonucleotides C2 and T1. Note that C3 contains the sequence of C1, except that a G is now present instead of G (Table I). The 2.3-kbp amplification fragment was digested with *Sal*I and *HindIII* and cloned into pYPBZ1, yielding pYCY3Z1.

When necessary, the above mentioned constructs (promoter plus open reading frame) were recovered by digestion with *Bam*HI and *HindIII* and placed in the multicopy plasmid YEplac181 (23). Mutations were performed in all cases by DNA sequencing and/or restriction mapping. The structure of the mentioned mutated forms is depicted in Fig. 1.

**Overexpression of PPZ1 in Yeast—** Strain YPH499 (19) was transformed with different constructs based in *pYES2* (Invitrogen), a high copy number plasmid containing the powerful GAL1 promoter element that is switched off when cells are grown on glucose and switched on when in galactose. To clone the wild type version of PPZ1, oligonucleotide C1, containing the first codons of PPZ1 and a *Bam*HI site, and oligonucleotide OV2 (Table I), corresponding to the sequence surrounding the stop codon, were used to amplify the PPZ1 open reading frame. The amplified fragment was digested with *Bam*HI and *Not*I and cloned into these same sites of pYES2. An NH2-terminal truncated version of PPZ1 was created by using oligonucleotides C2 (see above) and OV2. The amplification fragment was digested with *Sal*I and *Not*I and cloned into these sites of pBluescript SK−. This construct was digested with *HindIII* and *Not*I, and the insert cloned into *Smal*/*Not*I-digested pBluescript SK−. The insert was recovered by digestion with *HindIII*/*Not*I and cloned into pYES2 previously linearized with these restriction enzymes. Finally, the NH2-terminal half of PPZ1 was cloned into pYES2 as follows. Oligonucleotide OV3 (Table I) was used with oligonucleotide OV1 in PCR reactions to amplify a DNA fragment encoding from the initiating Met to PPZ1 to residue 319 (a stop codon, as well as a * HindIII site, have been included in OV3). The amplification fragment was digested with *Bam*HI and *HindIII* and cloned into plasmid pSP72 (Promega). The insert was recovered by digestion with * Smal* and * HindIII* and cloned into pBluescript SK−, previously linearized with these same enzymes. Finally, the insert was recovered by digestion

2 J. Clotet and J. Arin˜o, unpublished observations.
RESULTS

Gly-2 Can Be Myristoylated in Vivo and Is Relevant for Function in Salt Tolerance—Since PPZ1 contains a Gly-2 residue that is located within a consensus sequence for N-myristoylation, we explored the possibility that PPZ1 could be myristoylated in vivo and, in this case, if this modification could have functional relevance. Wild type DBY746 and ppz1Δ yeast cells were incubated with \(^{3}H\)myristate and extracts prepared and subjected to SDS-PAGE. Electrophoretic analysis of whole extracts (not shown) did not reveal any radioactive protein that could be assigned to PPZ1. However, when extracts from prelabeled wild type and ppz1Δ cells were incubated with anti-PPZ1 antibodies, incorporation of radioactivity in immunoprecipitated PPZ1 was found (Fig. 2A). Moreover, when immunoprecipitation was carried out from ppz1Δ cells harboring a construct that expressed a version of PPZ1 in which Gly-2 is mutated to Ala, we did not find radioactive incorporation into the protein. This was indicative that PPZ1 could be myristoylated in vivo and Gly-2 wild type, ppz1Δ and ppz1Δ cells carrying a low copy plasmid bearing the Gly → Ala version of PPZ1 expressed from the PPZ1 promoter were then challenged with different concentrations of lithium (Fig. 2, B and C). As described, at high lithium concentrations, wild type cells cannot grow while the ppz1Δ mutant survives. Expression of the wild type form of PPZ1 from plasmid pYC121 fully restores salt sensitivity, demonstrating that the wild type construct perfectly complements the chromosomal gene deletion. On the contrary, mutation of Gly-2 results in near complete loss of function, without affecting the amount of PPZ1 protein present in the cell, as determined by immunoblot experiments (not shown) nor the protein phosphatase activity of the enzyme when expressed in E. coli (see below).

The NH\(_2\)-terminal Half of PPZ1 Is Required for Function in Salt Homeostasis—Since the NH\(_2\)-terminal half of PPZ1 is a very distinctive feature of this phosphatase, we decided to investigate whether or not this part of the protein is necessary for function. This was made by generating several constructs based in a centromeric plasmid that, in low copy number, expressed from the PPZ1 promoter different versions of the protein based on modifications at the NH\(_2\) terminus. One of them, pYC4Z1, lacks a rather large segment located very close to the NH\(_2\) terminus (residue 17–190). A second construct (pYC5Z1) has a relatively short deletion near the COOH-terminal half (from residues 241–318). Both deletions closely correspond to regions that are well conserved between PPZ1 and PPZ2. Finally, in construct pYC2Z1, the complete NH\(_2\)-terminal half

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Expression of Wild Type and Mutated Forms of PPZ1 in E. coli and Determination of Protein Phosphatase Activity—Plasmid pYC1Z1, pYC2Z1, and pYC3Z1 were digested with SaI and HindIII, and the inserts were cloned into plasmid pSP72. The resulting constructs were digested with BamHI and PvuII, and each insert was ligated into the BamHI and SmaI sites of plasmid pGEX-KT (26), yielding pGEX-KT/C1, pGEX-KT/C2, and pGEX-KT/C3, respectively. The construct containing the Arg-451 to Leu mutation was constructed as follows: pGEX-KT/C1 was digested with ClaI and ligated with the 0.4-kbp ClaI fragment of PPZ1 that contained the desired mutation (see above). Positive clones with the appropriate orientation of the ClaI fragment (pGEX-KT/C6) were selected and used in expression experiments. Expression and purification of the different forms of PPZ1 in E. coli were carried out as described previously (7). Phosphatase activity was determined using \(^{32}P\)-labeled myelin basic protein as substrate in the presence of Mn\(^{2+}\) ions as described (7).

Expression and purification of the different forms of PPZ1 in E. coli were carried out as described previously (7). Phosphatase activity was determined using \(^{32}P\)-labeled myelin basic protein as substrate in the presence of Mn\(^{2+}\) ions as described (7).
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has been deleted, and a 348-residues protein that can be aligned with the catalytic subunit of type 1 or 2A phosphatase is expressed. The ppz1Δ strain D790-3A was transformed with the constructs and plated in medium containing different concentrations of lithium chloride, and growth was scored after 3 days of incubation at 28 °C (B). C, the same strains were grown for 19 h in liquid YPD medium containing 120 mM lithium chloride, and growth was scored by measuring the optical density of the culture at 660 nm. Data are represented as a percentage relative to the growth of the ppz1Δ strain D790-3A and correspond to the mean ± S.E. of three experiments.

FIG. 2. In vivo myristoylation of PPZ1. A, wild type DBY746 (PPZ1), JA-31 (ppz1Δ), and JA-31 carrying construct pYCZ1 (G→A) cells were grown. Aliquots received 0.33 mCi of [3H]myristate, and PPZ1 was immunoprecipitated from extracts. Radioactive PPZ1 was devoid of phosphatase activity. To this end, Arg-451 (which dressed this question by expressing a mutated version of PPZ1 with the constructs and growth on plates (A) or liquid medium (B) and growth evaluated as described in the legend of Fig. 2. Data are expressed as mean ± S.E. of four experiments.

Fig. 3. Effect of different deletions within the NH$_2$-terminal half of PPZ1 in salt tolerance. A, wild type D790-3D strain as well as the ppz1Δ strain D790-3A were transformed with the indicated constructs and growth on plates (A) or liquid medium (B) and growth evaluated as described in the legend of Fig. 2. Data are expressed as mean ± S.E. of four experiments.
The would correspond to Arg-96 in the catalytic subunit of rabbit skeletal muscle PP-1) was changed to Leu to yield plasmids pGEX-KT/C6 and pYC6Z1. In our case, expression of the mutated form of PPZ1 in E. coli (Fig. 4) shows that the modified protein is virtually inactive as phosphatase. It is worth noting that removal of the NH$_2$ terminus or mutation of the myristoylatable Gly-2 has a very limited effect on the phosphatase activity. As it is the case for the complete PPZ1, none of the mutated forms of PPZ1 displayed significant activity against 32P-labeled glycogen phosphorylase (not shown). When the Arg-451 to Leu mutation is expressed in yeast cells (Fig. 5, A and B), it is observed a failure to decrease the salt tolerance of a ppz1 disruption mutant, indicating that the protein is not functional in vivo. This failure was also observed when the construct was expressed from a multicopy plasmid (not shown). These results clearly confirms that the phosphatase activity of PPZ1 is essential for its function in salt tolerance.

Since it has been reported that overexpression of PPZ1 partially complements the temperature-sensitive phenotype of a mpk1Δ mutant, we decided to test whether or not the Arg-451 to Leu mutation could abolish this effect. When expressed from a multicopy plasmid, our wild type version of PPZ1 fairly complemented the temperature sensitivity of a mpk1Δ strain, whereas the construct mutated at Arg-451 completely failed to do so. The remaining constructs gave limited but detectable growth compared with wild type PPZ1 (not shown). This provided further evidence that mutation of a residue involved in catalytic activity completely abolish PPZ1 function.

Differential Effect of Mutations at the NH$_2$ Terminus of PPZ1 in Salt Tolerance and Caffeine Hypersensitivity—In addition to display an increased tolerance to salt, ppz1Δ mutants undergo cell lysis at relatively low concentrations of caffeine. We have tested all the PPZ1 mutants described above for caffeine hypersensitivity. In this case, wild type cells are more resistant to caffeine that ppz1Δ cells. As it can be observed in Fig. 2, both elimination of the NH$_2$ terminus or mutation of Arg-451, produce a nonfunctional PPZ1, exactly as observed when cells are challenged with salt. However, removal of residues 17–193 clearly affects the ability of PPZ1 to complement the ppz1 deletion, whereas the PPZ1 version lacking residues 241–318 still provides full complementation. This is exactly the opposite behavior to the one observed under salt stress. Moreover, change of Gly-2 to Ala yields a fully functional protein when cells are tested for caffeine stress, although it was essentially unable to restore normal salt tolerance (see Fig. 2).

PPZ1 Is Not Present in the Soluble Cytoplasmic Fraction—Immunoblot experiments using yeast cell extracts show that PPZ1 has an anomalous mobility on SDS-PAGE, corresponding to an apparent molecular mass of 91,000 Da. This is about 14,000 Da higher than the expected from the translation of its
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Fig. 4. Protein phosphatase activity of mutated forms of PPZ1 expressed in E. coli. The wild type form of PPZ1 (pGEX-KT/C1), the COOH-terminal half (pGEX-KT/C2), as well as the Gly-2 to Ala (pGEX-KT/C3) and Arg-451 to Leu (pGEX-KT/C6) mutations were expressed in E. coli as glutathione S-transferase fusion proteins. Extracts were prepared and their protein phosphatase activity measured as described (7) using $^{32}$P-labeled myelin basic protein as substrate. Data are expressed as mean ± S.E. of three experiments.

Fig. 5. Effect of the Arg-451 to Leu mutation on PPZ1 function in salt tolerance. Plate and liquid cultures were carried out as described in the legend of Fig. 2. Data are expressed as mean ± S.E. of four experiments.

open reading frame. A large amount of the protein (50–60%) is recovered in 25,000 × g pellets, whereas PPZ1 is virtually undetectable in 100,000 × g supernatants (Fig. 7A). Attempts to solubilize the protein under different conditions were unsuccessful in most cases and significant amounts of PPZ1 (never higher than 40–45%) were only recovered in the presence of a relatively high salt concentration (0.5 M NaCl). The fact that the protein is not solubilized using non-ionic detergent, chaotropic agents, or high pH suggests that most probably it is not directly attached to membranes. We have been able to detect by immunoblot the expression of the mutated versions of PPZ1 (with the only exception of pYCZ1). In all cases, the amount of the expressed proteins, as well as the pattern of distribution after centrifugation at 25,000 or 100,000 × g, is very similar to than observed for wild type cells (not shown). The failure to detect the COOH-terminal half of PPZ1 can be attributed to the fact that our antibodies essentially recognize determinants located at the NH$_2$-terminal region of PPZ1. This will also explain why the antibodies do not recognize PPZ2, which is only 43% identical to PPZ1 within the NH$_2$-terminal half, despite being almost 95% identical in its COOH-terminal half. Immunoblots using anti-PPZ1 antibodies indicates that a significant amount of the protein can be recovered with yeast nuclear fractions (Fig. 7B) and that the protein does not fractionate with plasma membranes (not shown).

Overexpression of the Phosphatase Activity of PPZ1 Results

Fig. 6. Effect of the different mutations of PPZ1 on the caffeine hypersensitivity phenotype. A, wild type DL790-3D and DL790-3A strain (App7) were transformed with the indicated constructs and plated on minimal medium plates (lacking leucine) containing different concentrations of caffeine. Growth was scored after 3 days of incubation at 28°C.

Fig. 7. Immunodetection and solubilization of PPZ1. A, extracts from wild type yeast cells were prepared as described and incubated under the mentioned conditions for 20 min at 4°C. Samples were then centrifuged at 100,000 × g for 45 min and supernatants (S) and pellets (P) subjected to SDS-PAGE and immunoblot. B, spheroplasts from wild type DBY746 and JA-31 (ppz1Δ) cells were made and extracts prepared (left). Intact nuclei were isolated from strain DBY746 by differential centrifugation using the long protocol described in Ref. 27. Final pellet (P) and supernatant (S) were analyzed by immunoblot (center). Isolated nuclei were lysed and samples centrifuged at 100,000 × g for 45 min. Supernatant (S) and pellet (P) were analyzed by immunoblot using antibodies against PPZ1 (right). Filters were stripped and incubated with antibodies against NOP1, a nuclear protein that is recovered in the so-called nuclear envelope fraction (28).

in Inhibition of Growth—Several lines of reasoning lead us to the idea that the activity of PPZ1 might be strictly controlled in yeast cells. For instance, we have observed that the expression of PPZ1 from its own promoter in high copy number significantly reduces growth rate of wild type cells. To test the effect of a strong overexpression of PPZ1, we made a construct, based in a multicopy plasmid, that has the entire PPZ1 open reading frame driven by the strong and regulatable GAL1 promoter. This construct was used to transform wild type yeast cells. As it can be observed in Fig. 8, expression of PPZ1 from the GAL1 promoter results in a dramatic decrease in growth in both plate and liquid cultures. Interestingly, the overexpression of the COOH-terminal half of PPZ1 results in an equally dramatic phenotype, whereas overexpression of the NH$_2$-terminal half does not affect growth at all. Cells overexpressing the NH$_2$-terminal half of PPZ1 did not show changes in tolerance to salt or sensitivity to caffeine when compared with wild type cells (not shown).
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The finding that PPZ1 is involved in the maintenance of cell integrity (9, 10) as well as in the control of salt tolerance (11) indicates that this novel phosphatase represents a key component of yeast cells. In addition, the unusual primary structure of the protein suggests the possibility of unsuspected regulatory mechanisms. This idea prompted us to undertake a study, based on the expression of mutated forms of PPZ1, to gain insight into the biological role of this protein.

One of the most relevant features of the NH$_2$-terminal region of PPZ1 (and of PPZ2) is the presence of a consensus sequence for N-myristoylation (5). Studies using synthetic peptides have shown that the amino-terminal sequence of PPZ1 (as well as of PPZ2) can be a substrate in vitro for the yeast N-myristoyltransferase, the enzyme responsible for N-myristoylation (29). We show here that this covalent modification might be possible in vivo and that mutation of Gly-2 prevents myristoylation. A possible explanation for the relatively low incorporation of radioactive myristate into PPZ1 might be that only a subpopulation of the protein becomes myristylated under our experimental conditions. This situation has been described previously for the GPA1 gene product, a Go subunit coupled to the yeast pheromone receptor (30). However, whereas myristylated and unmyristylated species of GPA1 are easily resolved by SDS-PAGE, we always observe a single PPZ1 band (although, as described above, with anomalous mobility). In any case, our data indicate that the presence of Gly-2 is important for PPZ1 function in salt homeostasis, whereas it is dispensable under caffeine stress and suggest that myristoylation of this residue might have functional relevance. This finding is important, because change or deletion of the myristoylatable Gly does not always result in clear functional consequences. For instance, the regulatory subunit of calcineurin encoded by the gene CNB1 (15–17) is myristoylated in vivo (6). However, mutation of its myristoylatable Gly-2 to Ala yields a functional protein (31, 32), indicating that, in this case, myristoylation is apparently dispensable for proper function. Myristate appears to be important in mediating protein-protein and/or protein-membrane interactions, although accessory factors or other covalent modifications are often needed. In our case, since PPZ1 is not a soluble protein, an attractive hypothesis would be that the biological effects due to lack of myristoylation might be derived from an alteration of the normal subcellular distribution of the enzyme. However, this is probably not the case, since we have observed that wild type and nonmyristoylatable PPZ1 are recovered in the same fractions when extracts are subjected to centrifugation or fractionation in sucrose gradients (not shown).

It must be noted that mutation of Gly-2 does not directly result in significant changes in phosphatase activity, as deduced from the bacterial expression of recombinant PPZ1. From this, one might conclude that this change could affect the function without altering the catalytic activity of PPZ1. Although this might be the case, the possibility that, in yeast cells, myristoylation might be responsible for fruitful interactions that would be necessary for full PPZ1 catalytic activity cannot be ruled out. Interestingly, we have recently cloned a gene from Schizosaccharomyces pombe encoding a putative PPZ homolog, which is 78% identical to PPZ1 within the COOH-terminal region (whereas only 62% identical to PP-1), and retains the myristoylatable NH$_2$-terminal glycine.

The importance of the catalytic activity of PPZ1 for proper function has been demonstrated by expressing a PPZ1 version mutated at Arg-451. This residue is highly conserved in many eukaryotic Ser/Thr protein phosphatases, and it is postulated to be involved in the catalytic mechanism of the enzyme (33, 34). Furthermore, mutation of the equivalent residue in the phosphatase encoded in the genome of the lambda phage results in a dramatic loss of catalytic activity when expressed in bacteria (35). The Arg-451 to Leu mutation also yields a recombinant PPZ1 that has no phosphatase activity, and therefore, it is most likely to produce a catalytically inactive PPZ1 when expressed in yeast cells. This is in agreement with the finding that the mutated protein is unable to restore the wild type phenotype either under salt or caffeine stress, even when expressed from a high copy number plasmid. Furthermore, under these same conditions, the mutated protein completely fails to complement the temperature sensitivity of a mpk1Δ strain. Therefore, it is concluded that the catalytic activity is an absolute requirement for PPZ1 function. Since the bacterially expressed COOH-terminal half of PPZ1 displays protein phosphatase activity similarly to the complete PPZ1, including the inability to dephosphorylate glycogen phosphorylase (7), it is unlikely that the loss of PPZ1 function as a result of removal of the NH$_2$-terminal region would be due to complete loss of the catalytic activity of the protein.

From our data, it is clear that an entire NH$_2$-terminal region, as well as a functional protein phosphatase moiety, are necessary for normal PPZ1 function and that the phosphatase activity is necessary but not sufficient for PPZ1 function. A most interesting finding is that the NH$_2$-terminal half of PPZ1 contains several structural determinants that are specific for a given function. For instance, the possibility of the protein to undergo in vivo N-myristoylation, as well as the presence of a relatively short region (from residue 241 to 318) of the NH$_2$-terminal half, are needed for function in the salt tolerance pathway but dispensable in the maintenance of cellular integrity, a pathway that links PPZ1 to the protein kinase COOH-activated MAP kinase MPK1/SLT2 (9, 10). Conversely, determinants necessary for full function in the later pathway are dispensable in the former one. This is in agreement with the notion that both pathways, although undoubtedly linked

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3 L. Baleells and J. Arinó, unpublished results.
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through the PPZ phosphatases, may be otherwise unrelated. It should be noted that, whereas the catalytically inactive protein is not functional at all in yeast even when expressed from a multicopy plasmid, under these conditions the COOH-terminal construct is able to partially complement a ppz1Δ mutant under both caffeine salt tolerance stresses (not shown). This suggest that the lack of the NH2 terminus can be suppressed up to some extent by an excess of the phosphatase moiety. These results are in agreement with the notion that the NH2-terminal half of PPZ1 might have a (positive) regulatory role of the essential phosphatase activity of the protein and that it could be considered as a built-in regulatory subunit with several functional domains. Although the observation that, after centrifugation, mutated forms of PPZ1 lacking substantial fragments of the NH2 terminus show a distribution similar to than of the wild type protein might suggest that this region is not involved in subcellular targeting of PPZ1, the possibility that the NH2-terminal region might be responsible for functionally relevant interactions with other proteins cannot be ruled out.

Overexpression of the complete PPZ1 and of its COOH-terminal half under the GAL1 promoter results in an intense growth defect, which is not observed when overexpressing the NH2-terminal half. This is suggestive that the growth defect might be due simply to a general increase in Ser/Thr phosphatase activity and not necessarily to an excess of function specific of PPZ1. It should be kept in mind that the catalytic COOH-terminal moiety of PPZ1 is rather similar (about 60% of identity) to the catalytic subunit of yeast PP-1, encoded by the DIS2S1/GLC7, and that there is ample evidence that some extent by an excess of the phosphatase moiety. These results are in agreement with the notion that the NH2-terminal region might be responsible for functionally relevant interactions with other proteins cannot be ruled out.

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