Psrlp/Psr2p, two plasma membrane phosphatases with an essential DXDX(T/V) motif required for sodium stress response in yeast

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Running title: Novel plasma membrane regulators of sodium stress
SUMMARY

Regulation of intracellular ion concentration is an essential function of all cells. In this study, we report the identification of two previously uncharacterized genes, **PSR1** and **PSR2**, that perform an essential function under conditions of sodium ion stress in the yeast *Saccharomyces cerevisiae*. Psr1p and Psr2p are highly homologous and were identified through their homology with the ER membrane protein Nem1p. Localization and biochemical fractionation studies show that Psr1p is associated with the plasma membrane via a short amino-terminal sequence also present in Psr2p. Growth of the **psr1psr2** mutant is severely inhibited under conditions of sodium, but not potassium ion or sorbitol stress. This growth defect is due to the inability of the **psr1psr2** mutant to properly induce transcription of **ENA1/PMR2**, the major sodium extrusion pump of yeast cells. We provide genetic evidence that this regulation is independent of the phosphatase calcineurin, previously implicated in the sodium stress response in yeast. We show that Psr1p contains a DXDX(T/V) phosphatase motif essential for its function *in vivo* and that a Psr1p-PtA fusion, purified from yeast extracts exhibits phosphatase activity. Based on these data, we suggest that Psr1p/Psr2p, members of an
emerging class of eukaryotic phosphatases, are novel regulators of salt stress response in yeast.
INTRODUCTION

Response and adaptation to rapidly changing environmental conditions is a fundamental feature of all living cells. Because of the knowledge of its sequenced genome and the availability of powerful genetic approaches, budding yeast *Saccharomyces cerevisiae* has been a particularly valuable model system to study such responses. Of particular significance for this unicellular eukaryote is the response to changes in osmolarity of the medium. Yeast cells are able to detect and respond to changes in osmolarity by two independent osmosensors, Sln1p and Sho1p (1, 2). These membrane-bound proteins activate the so-called high osmolarity glycerol (HOG) MAP kinase cascade (1, 3), that in turn mediates the transcriptional activation of several genes required for adaptation to high osmolarity mostly through the stress response elements (STREs). These include several general osmotic stress genes, like *GPD1* (glycerol phosphate dehydrogenase) required for prevention of water loss, heat shock proteins like *HSP12*, or *CTT1* (cytosolic catalase) among others (4-6).

The HOG pathway is also responsible for transmitting stress signals emerging from high concentrations of certain ions, such as lithium or sodium (7). High salinity media can be toxic for yeast cells since they
lead to loss of turgor pressure and block several metabolic reactions. In this case, an essential event required for the survival of yeast cells is the transcriptional activation of the ENA1/PMR2 gene. ENA1/PMR2 encodes for a P-type ATPase required for the efflux of sodium ions (8, 9). Although yeast cells contain a tandem array of nearly identical genes encoding ion pumps involved in sodium tolerance, only the first of them, ENA1/PMR2, is strongly induced and therefore required during sodium ion stress (10).

Apart from the HOG-dependent induction during sodium ion stress, PMR2 transcription is also induced by another independent pathway mediated by the calcium/calmodulin dependent phosphatase calcineurin (11, 12). Calcineurin function is exerted through the dephosphorylation and subsequent nuclear translocation of the transcription factor Crz1p (13, 14). Finally, basal and sodium induced levels of PMR2 can be regulated by the Ser/Thr phosphatase Ppz1p and its regulatory subunit HAL3 (15, 16) or by glucose and nitrogen metabolism (7, 17, 18).

In the present work, we show that two previously uncharacterized plasma membrane proteins, Psr1p and Psr2p, are essential for an efficient sodium ion stress response through transcriptional activation
of the major sodium extrusion pump of yeast cells, *PMR2*. Our data suggest that Psr1p/Psr2p regulate *PMR2* transcription through a pathway that is independent of the one mediated by calcineurin. Psr1p and Psr2p contain a DXDX(T/V) motif within their conserved C-terminal domain that has been recently identified in several phosphohydrolases and in Fcp1p, the essential phosphatase of RNA polymerase II (19,20). We demonstrate here that the DXDX(T/V) motif of Psr1p is essential for its function in sodium stress response and that native Psr1p exhibits phosphatase activity *in vitro*. Finally we suggest that the function of the Psr1p/Nem1p homology domain may not be restricted to the DXDX(T/V) motif-dependent phosphatase activity.
EXPERIMENTAL PROCEDURES

Yeast strains, microbiological techniques, plasmids and DNA manipulations

The yeast strains used in this work are listed in Table I. Standard DNA manipulations (restriction analysis, ligation, PCR amplification and DNA sequencing) were performed as described earlier (21). Microbiological techniques (growth and transformation of yeast and E. coli strains, plasmid recovery, mating, and tetrad analysis) were done as described in (22). The following plasmids were used: YCplac111, ARS1/CEN4 vector with the LEU2 marker (23); YEplac181, 2µ vector with the LEU2 marker (23); pFR70, 2µ vector expressing the ENA1-LacZ reporter (6, 24); pJQ10, 2µ vector expressing ENA1/PMR2 under the control of the PGK1 promoter (25).

Deletion of the PSRI and PSR2 genes

In order to knock-out PSRI and PSR2, the complete ORF of these genes was deleted by generating two unique BamHI restriction sites via PCR-mediated mutagenesis, one just after at the ATG start codon and the other just before the stop codon, removal of the DNA between start and
stop codon and insertion of a BamHI DNA restriction fragment containing either the \textit{HIS3} (for the construction of \textit{psr1::HIS3}) or \textit{TRP1} (for the construction of \textit{psr2::TRP1}) marker. The \textit{psr1::HIS3} and \textit{psr2::TRP1} alleles, each containing 5’ and 3’ non-coding flanking regions required for homologous recombination, were transformed into a haploid RS453 wild-type strain. In both cases, correct integration of the disrupted gene copy at the homologous gene locus was verified by PCR analysis. To construct the \textit{psr1 psr2} double deletion mutant, a \textit{psr1::HIS3} strain carrying the p-\textit{URA3-PSR1} plasmid was transformed with a \textit{psr2::TRP1} allele. Correct transformants grew as wild-type on plates containing 5-FOA, showing that the \textit{psr1 psr2} mutant does not exhibit any growth defects at standard growth conditions.

\textbf{Spot dilution growth assays}

8 µl of serial dilutions of saturated yeast cultures were spotted onto the indicated media (YPD or YPD containing 1M sorbitol, 1M NaCl, 1M KCl and containing, where indicated, 1µg/ml FK506). Plates were incubated at 30°C, for 2 (YPD medium) or 5 (YPD containing 1M NaCl) days.
Construction of PSRI Fusion Genes and Mutants

In order to epitope-tag PSRI, a unique BamHI site was introduced just before the stop codon by PCR (…ATA.GGA.TCC.TAA...). A BamHI fragment encoding either two IgG binding domains from Staphylococcus aureus Protein A (380 bp) or the S65T/V163A variant of the Green Fluorescent Protein (GFP) (720 bp) was inserted in frame into the BamHI site generated at the stop codon of PSRI. All fusion proteins were expressed under the control of the PSRI promoter from centromeric vectors (YCplac111-LEU2-PSR1-PtA, YCplac111-LEU2-PSR1-GFP) or a 2μ vector (YEplac181-LEU2-PSR1-GFP). PtA and GFP-fusion proteins were functional since they could complement the growth defect of psr1psr2 cells at media supplemented with 1M NaCl (not shown).

To construct a fusion protein between the 28 amino-terminal residues of PSRI and GFP, two PCR fragments, one coding for the promoter and the 28 N-residues of Psr1p (M(1) to S(28)) followed by a BamHI site and a second one coding for a BamHI site followed by the PSRI stop codon and its 3’ UTR, were digested with SphI/BamHI and BamHI/EcoRI, respectively, and ligated into a SphI/EcoRI digested
YCplac111 vector. A BamHI GFP fragment was inserted in-frame at the unique BamHI site preceding the \textit{PSR1} stop codon.

To construct the D(263)E and D(265)E mutants of Psr1p, a silent mutation inserting a unique XbaI site in the codon preceding the two aspartic acids at positions 263 and 265 (underlined) of \textit{PSR1} was generated (\ldots ATT\textbf{CTA.GAC.CTG.GAT.GAA}\ldots). Oligonucleotide primers containing the D(263)E (\ldots ATT\textbf{.CTA.GAA.CTG.GAT.GAA}\ldots) or the D(265)E (\ldots ATT\textbf{.CTA.GAC.CTG.GAA.GAA}\ldots) mutations were used to amplify the region corresponding to the C-terminus of \textit{PSR1}, with a reverse primer introducing a BamHI site just before the stop codon (see above). XbaI/BamHI digested PCR fragments were ligated into a XbaI/BamHI cut YCplac111-LEU2 vector carrying the \textit{PSR1} gene with the BamHI site before the stop codon. For the deletion of the conserved C-terminal domain of Psr1p, two PCR fragments, one coding for the promoter and amino-terminal portion of Psr1p until E(226) followed by a BamHI site and a second one coding for a BamHI site followed by the \textit{PSR1} stop codon and its 3\textquotesingle UTR, were digested with SphI/BamHI and BamHI/EcoRI, respectively, and ligated into a SphI/EcoRI digested YCplac111 vector. In all cases, the Protein A tag was inserted in frame at the unique BamHI site preceding the \textit{PSR1} stop codon.
To construct the G2A and the C9G, C10G mutants of Psr1p, a silent mutation inserting a EcoRI site 16 codons downstream of the ATG was generated (...TCG.AAT.TCC...). Oligonucleotide reverse primers containing the G2A (...GAA.AGC.CAT...) or the C9G, C10G (...AGA.GCC.GCC.CAG...) mutations and a forward primer priming 600 nucleotides downstream of the ATG were used to amplify the promoter and the 19 amino-terminal residues of PSR1. A second PCR product was generated with a forward primer introducing the EcoRI site and a reverse oligo priming 700 nucleotides upstream of the ATG. The two PCR fragments were digested respectively by SphI/EcoRI and EcoRI/PstI and ligated into a Ycplac111-PSR1-PtA construct. All constructs were verified by DNA sequencing.

Affinity-Purification and phosphatase assay of Psr1p-PtA fusion proteins

For affinity-purification of the Psr1p-PtA fusions, yeast strains expressing the corresponding PtA fusion proteins (Table I) were grown and spheroplasted as previously described (26). 1g of frozen spheroplast pellet was lysed in 22 ml ice-cold lysis buffer (150 mM KCl,
20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1% Triton X-100) supplemented with a cocktail of protease inhibitors (Complete EDTA-free, Boehringer Mannheim). The extract was centrifuged for 15 min at 15,000 rpm (SS-34 rotor, Sorvall) and the supernatant was first pre-cleared by incubation with Sepharose Fast flow beads (Pharmacia), and then loaded onto a column packed with 100 µl IgG-Sepharose beads (Pharmacia). The column was washed with 40 ml of lysis buffer, 8 ml of wash buffer (1M NaCl, 20 mM Tris-HCl pH 8.0, 5 mM MgCl₂), and finally with 10 ml of phosphatase buffer (10 mM KOAc, 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 0.1 mM EDTA, 10% glycerol). The beads were then transferred to a 1ml Mobicol column (MoBiTec) and washed for 15 min with 1 ml phosphatase buffer at 30°C. 30 µl of beads were then incubated with 200 µl phosphatase buffer containing 10 mg/ml p-nitrophenylphosphate (Sigma) for 30 min at 30°C. The supernatant was collected by centrifugation and its absorbance was measured at 410 n.m.

**Extraction analysis of Psr1p-PtA**

To determine whether Psr1p is a membrane associated protein, spheroplasts were prepared from early log phase cultures of a PSR1-PtA
1 g of spheroplasts was lysed with a dounce homogenizer in 16 ml extraction buffer (150 mM KCl, 20 mM Tris-HCl, pH 8.0, 5 mM MgCl) and centrifuged at 100,000 g for 30 min at 4°C with a Beckman TLA 120.2 rotor. The insoluble pellet was resuspended in either 16 ml of extraction buffer containing 1% Triton X-100 or 1 M NaCl, or in 16 ml 0.1 M sodium carbonate pH 11.5. Extracts were incubated for 15 min on ice and then separated into a soluble and pellet fraction by centrifugation at 100,000 g for 30 min at 4°C. Equivalent amounts from all fractions were dissolved in SDS-sample buffer, heated at 95°C for 3 min and analyzed by SDS-PAGE, blotted onto nitrocellulose and probed with anti-PtA (1:3000, DAKO) or anti-Dpm1p (1:1000, Molecular Probes) antibodies. Lysis of the protein-A tagged G2A and C9G,C10G mutants of PSR1 was performed as described above, except that extracts were centrifuged at 70,000 g for 30 min at 4°C. SDS-PAGE and Western blot analysis were performed according to (21). All extractions were performed at 4°C in the presence of a protease inhibitor cocktail (Boehringer Mannheim).

**β-galactosidase assays**
For β-gal assays, cells growing in synthetic medium were diluted into YPD with or without 1M NaCl and grown for 6h at 30°C. 0.5 OD₆₀₀ of cells were spun down, permeabilized with chloroform and SDS and glass-bead lysed. 10 µl of the extracts were assayed as previously described (27). β-galactosidase activities were normalized to the protein concentration of each sample as measured using the Bio-Rad Bradford kit (Bio-Rad). Absolute values of β-galactosidase activity varied depending on the culture density. In each experiment, cells were grown to equivalent densities.
RESULTS

Psr1p and Psr2p belong to a large family of uncharacterized proteins from different species, that share a high degree of homology in a domain of 200 amino acid residues. This family includes Nem1p, an ER membrane protein which is required for nuclear/ER membrane morphogenesis in the budding yeast *Saccharomyces cerevisiae* (26). BLAST searches with Nem1p identified three uncharacterized yeast genes corresponding to ORFs YLL010c, YLR019w and YPL063w that share 36% identity over their entire 200 residue long C-termini with the corresponding domain of Nem1p. Interestingly, YLL010c and YLR019w are closely related to each other and each contains a putative lipid-attachment sequence at the N-terminus, suggesting that, like Nem1p, they are also membrane associated (Figure 1). To gain further insight into the function of the Nem1p family of proteins, we analyzed this pair of genes, designated *PSR1* (YLL010c) and *PSR2* (YLR019w, see below) in more detail.

**Psr1p localizes to the plasma membrane via a targeting signal present in its extreme N-terminus**
PSR1 and PSR2 encode acidic proteins of 427 (predicted MW 47.9 kDa, pI 4.9) and 397 (MW 44.7 kDa, pI 4.6) amino acid residues, respectively. Alignment of the two sequences shows that the proteins are almost identical over their conserved C-terminal portions (83% identity, 89% similarity) and more distantly related over their respective amino-terminal halves (29% identity, 45% similarity over their 175 N-terminal residues) (Figure 1B).

In order to determine its subcellular localization, Psr1p was tagged with the Green Fluorescent Protein (GFP) in its C-terminus. The fusion protein, expressed under the control of the authentic PSR1 promoter was functional (see later). When psr1 cells expressing the Psr1p-GFP fusion were observed in the fluorescence microscope, a clear and distinct plasma membrane labeling was seen (Figure 2). No intracellular staining was detected, suggesting that under steady state conditions Psr1p localizes mostly to the plasma membrane.

Hydropathy plot analysis indicated that, unlike their ER-related counterpart Nem1p, Psr1p and Psr2p do not contain any putative membrane-spanning sequence (Figure 3A). However, the 12 very amino-terminal residues of both Psr1p and Psr2p exhibit a high value of hydrophobicity on Kyte-Doolittle plots. Interestingly, this short
stretch, that is highly conserved in both proteins, contains a glycine, immediately after the starting methionine and two cysteines in positions 9 and 10, suggesting that Psr1p and Psr2p might be target of a lipid modification, possibly myristoylation and/or palmitoylation. In order to further investigate how Psr1p is associated with the plasma membrane, we used a strain expressing a Psr1p-Protein A (Psr1p-PtA) fusion from a centromeric plasmid under the control of the PSR1 promoter (see Materials and Methods). When spheroplasts from cells expressing Psr1p-PtA were lysed in a buffer lacking detergent, the Psr1p fusion was found mostly in the insoluble pellet (P100). Further fractionation of this insoluble Psr1p-PtA pool demonstrated that it can be efficiently solubilized in the presence of 1% Triton X-100, but not in 1M NaCl (Figure 3B) or 0.1M carbonate pH 11.5 (not shown). Very similar behaviour was exhibited by the membrane protein Dpm1p (Figure 3B). Therefore, although lacking a typical transmembrane domain sequence, Psr1p behaves as an integral membrane protein. Interestingly, a minor amount of a faster migrating Psr1p-PtA species (probably a breakdown product) appears to be enriched in the soluble fraction (Figure 3B).

To directly test whether the conserved N-terminus of Psr1p mediates its membrane binding, we constructed two mutants, PSR1-G2A and PSR1-
C9G,C10G, where the residues that could mediate the covalent attachment of myristoyl- and palmitoyl-groups, respectively, have been mutated. As seen in Figure 3C, the G2A mutation does not affect the membrane association of Psr1p since, in the absence of detergent, most of the mutant protein is still found in the pellet. However, the two cysteines at positions 9 and 10 are essential for the membrane binding of Psr1p, since the C9G,C10G mutant is found almost exclusively in the soluble fraction (Figure 3C). This result strongly suggests that palmitoylation of cysteines 9 and 10 is responsible for the plasma membrane association Psr1p.

Since lipid-modified sequences have been shown often to target proteins to the plasma membrane (28), the first 28 residues of Psr1p were fused to GFP and expressed in yeast. Indeed, we found that it was localized to the plasma membrane in a way that is indistinguishable to that of the full-length Psr1p-GFP (compare plasma membrane staining in Figures 2 and 3D). We therefore conclude that Psr1p is targeted to and associated with the plasma membrane via a conserved amino-terminal sequence, which is also present in Psr2p.

The complex migration of Psr1p-PtA on SDS-PAGE suggests that, apart from the lipid attachment, Psr1p may be target of additional post-
translational modifications. Indeed, Psr1p appears to be phosphorylated in its N-terminal portion, since when affinity-purified Psr1p-PtA is treated with alkaline phosphatase, the doublet of each one of the two Psr1p forms along with its smeary appearance disappears and instead, a single low and high molecular weight form appear (Figure 3E). Since the Psr1ΔCp-PtA mutant exhibits a similar shift in its molecular weight forms, at least some of the putative phosphorylation site(s) must be located within the N-terminal half of the protein.

**Psr1p and Psr2p perform an essential function in sodium ion stress response**

To investigate the function of Psr1p and Pr2p, we deleted the *PSR1* and *PSR2* genes. Cells lacking either gene alone were viable and able to grow normally at both 16 and 37°C (data not shown). Since the two proteins exhibit such a high homology over their C-terminal domains, cells in which both genes were deleted were also generated. Although no growth defects were observed under standard media, the growth of the double, but not single, mutant was severely inhibited in media containing 1M NaCl (Figure 4) or 0.3M LiCl (data not shown). This phenotype did not appear to be due to a general osmotic defect, as,
unlike the *bona fide* osmosensitive mutant *hog1*, no growth inhibition was observed when the *psr1psr2* mutant was grown in the presence of 1M sorbitol or 1M KCl (Figure 4). Furthermore, the *psr1psr2* mutant grew as wild-type on plates containing 0.4M CaCl$_2$, 0.5mg/ml calcofluor white, 5mM caffeine or 20µg/ml geneticin (data not shown). To confirm the specificity of the growth defect on high salinity media, a single *psr1psr2* double deletion mutant was transformed with either two empty centromeric vectors, or with the same vectors carrying either *PSR1*, *PSR2*, or both genes. Cells lacking any of the *PSR* genes did not grow on YPD plates containing 1M NaCl whereas *PSR1* or *PSR2* cells did (not shown). Based on these data we conclude that Psr1p and Psr2p perform a redundant function which is essential under conditions of sodium ion stress. We therefore named the corresponding genes *PSR1* and *PSR2* (for *P*lasma membrane *S*odium *R*esponse 1 and 2).

**Psr1p and Psr2p induce *ENA1/PMR2* expression under conditions of sodium ion stress**

Since many mutants with a primary defect in secretion and/or cytoskeletal organization are also osmotically sensitive, and since Nem1p, the only characterized member of the Psr family, shows severe
defects in the nuclear/ER membrane organization (26), we first analyzed whether the psr1psr2 mutant exhibits other defects that could indirectly cause sodium sensitivity. The morphology of the psr1psr2 mutant was indistinguishable from that of the isogenic wild-type control when observed at the ultrastructural level by thin section electron microscopy, (data not shown). Furthermore, the psr1psr2 mutant does not show any defect in cell wall integrity (calcofluor staining), actin cytoskeleton (phalloidin staining) or endocytosis (uptake of FM4-64) (data not shown). These data suggest that the psr1psr2 mutant might be specifically involved in a sodium stress response pathway.

Different signal transduction pathways responsible for adaptation of yeast to high sodium and lithium concentrations converge to the transcriptional activation of ENA1/PMR2, the major sodium/lithium extrusion pump of yeast cells. We therefore tested whether constitutive expression of PMR2 can rescue the growth defect of the psr1psr2 mutant during salt stress. Indeed, we found that overexpression of PMR2 from the PGK1 promoter suppresses the sodium-induced growth defect of the psr1psr2 mutant (Figure 5A). These data suggest that the growth defect of the psr1psr2 mutant on
high salinity media is due to its inability to properly induce transcription of \textit{PMR2}.

To address this possibility directly, we used a \textit{PMR2} promoter-lacZ reporter (7, 24) to follow \textit{PMR2} expression levels during sodium stress. The \textit{psr1psr2} mutant, or the isogenic wild-type control (\textit{PSR1PSR2}), were transformed with the pmr2::LacZ reporter gene and grown in media supplemented with 1M NaCl for 6 hr (Figure 5B). As previously reported, wild-type cells that were challenged with 1M NaCl induced \textit{PMR2} expression. However, in the \textit{psr1psr2} mutant, sodium-dependent induction of the pmr2::LacZ reporter was reduced to half of the wild-type levels (Figure 5B). This reduction appears to affect primarily the induced levels of \textit{PMR2}. These data suggest that Psr1p and Psr2p are upstream mediators of \textit{PMR2} expression upon sodium stress.

**Psr1p/Psr2p function is independent of the calcineurin sodium stress response pathway**

We next analyzed whether Psr1p and Psr2p are functionally linked with any of the previously described salt stress response pathways. We reasoned that Psr1p/Psr2p are not required for the general HOG-
mediated osmotic stress pathway, since the *psr1psr2* defect is manifested only in NaCl, but not other high osmolarity media, like sorbitol, or KCl, which have been shown to activate the Hog1p-dependent pathway.

A second and independent mediator of *PMR2* induction during sodium stress in yeast is the type B calcium dependent phosphatase calcineurin (7, 11, 12, 29). Since calcineurin function can be specifically inhibited by the drug FK506 in yeast, as well as in mammalian cells (30), we tested the growth properties of the *psr1psr2* mutant under a mild salt stress (0.4M NaCl), in the presence or absence of FK506. As seen in Figure 6A, the *psr1psr2* mutant has a slight growth defect in the absence of the drug, whereas upon FK506-mediated calcineurin inhibition, it is unable to grow. This shows that the effects of the lack of the *PSR* genes and the inactivation of calcineurin, in respect to growth on high salinity media, are additive. Therefore, Psr1p/Psr2p and calcineurin appear to activate *PMR2* transcription through parallel pathways.

To address this possibility directly, we followed sodium-dependent induction of the pmr2::LacZ reporter in the presence or absence of FK506, in *psr1psr2* or wild-type cells (Figure 6B). In agreement with
previous reports, FK506 decreases PMR2 induction in wild-type cells. Deletion of PSR1/PSR2 further decreases, but does not completely eliminate sodium-dependent induction of the pmr2::LacZ reporter. These data indicate that PSR1/PSR2-dependent activation of PMR2 expression does not overlap with the calcineurin function in sodium stress response.

**Psr1p contains a DXDX(T/V) phosphatase motif which is essential for its function in vivo**

Apart the different closely related homologues of the Psr1p/Nem1p family, it has been recently reported that both the yeast and the human orthologues of the FCP1 gene product exhibit sequence similarity with a conserved domain present in several proteins which includes the C-terminal domains of Psr1p/Psr2p and Nem1p (31). Yeast Fcp1p has been recently shown to function as the major phosphatase that dephosphorylates the RNA polymerase II large subunit C-terminal domain (Kobor et al 1999). The corresponding conserved area in Fcp1p is clearly shorter and not as homologous as that found in the other members of Nem1p family (see Figure 9). However, Kobor *et al* found a short stretch of 11 residues within the
conserved portion of Fcp1p, initially identified as the phospho-acceptor site, present in several phospho-hydrolases and -transferases (19). This stretch is essential for the function of the protein in vivo and in vitro (20). We therefore tested whether the corresponding motif in Psr1p is required for its function in the sodium stress response. Since it was shown that the first aspartate of the DXDX(T/V) acts as the phosphoryl acceptor residue (19), we used site-directed mutagenesis in order to change the two conserved aspartates of the D(263)-L-D(265) phosphatase motif into glutamates. In order to compare the effect of these single point mutations with the lack of function of the entire conserved domain, we also constructed a Psr1ΔCp mutant lacking the entire conserved C-terminal domain of Psr1p. All mutants were tagged C-terminally with protein A and transformed into the psr1psr2 mutant. As seen in Figure 7, the C-domain encodes an essential function for Psr1p, since the Psr1ΔCp mutant is not able to rescue the growth defect on the psr1psr2 mutant on high salinity media. Moreover, neither the D263E nor the D265E allele of PSRI were able to complement the psr1psr2 mutant upon sodium stress. This was not due to instability or degradation of the mutant proteins, since western blot analysis demonstrated that they are expressed at similar levels as the wild-type Psr1p-PtA (data not shown). These data show that Psr1p contains a DXDX(T/V) motif that is essential for its function.
in sodium stress response and suggest that Psr1p might also possess phosphatase activity.

**Psr1p has phosphatase activity *in vitro***

In order to directly address whether Psr1p has phosphatase activity, we isolated the native yeast protein from the *psr1psr2* cells and assayed it against the artificial phosphatase substrate p-nitrophenylphosphate (p-NPP). We used IgG-Sepharose chromatography to affinity purify, either functional wild-type Psr1p-PtA, the C-terminal truncation mutant Psr1ΔCp-PtA and the Psr1(D263E)p-PtA or Psr1(D265E)p-PtA point mutants. All Psr1p fusion proteins were expressed under the control of their authentic promoters from high-copy vectors. Coomassie staining showed that the various fusion proteins appear essentially pure. The major protein band appearing in the 40 kDa range in the wild-type and point mutant Psr1p fractions corresponds to a breakdown product (compare Coomassie staining and western blot in Figure 8A). As a control for the phosphatase assay, the PtA tag alone was expressed and purified from the same *psr1psr2* cells in parallel with the various Psr1p fusions.
As shown in Figure 8B, native wild-type Psr1p-PtA isolated from yeast extracts, hydrolyzes p-NPP to produce p-nitrophenol that absorbs at 410 nm. Importantly, the Psr1ΔCp-PtA fusion gave very low values showing that the conserved C-domain of Psr1p is required for the phosphatase activity, as measured by the hydrolysis of pNPP. We find that, under the conditions used in our assay, approximately 1µg of native Psr1p-PtA produces 1nmol of p-nitrophenol per 30 min. Interestingly, both the D(263)E and D(265)E point mutants of Psr1p, although not able to functionally complement the psr1psr2 mutant, appear to have a low residual phosphatase activity (Figure 8B, see Discussion).

**DISCUSSION**

In this study, we report the identification and characterization of *PSR1* and *PSR2*, two novel and functionally redundant phosphatases in *Saccharomyces cerevisiae* that perform an essential role under conditions of sodium ion stress. Both genes were initially identified through their homology with the conserved ER membrane protein Nem1p (26). *PSR1* and *PSR2*, along with a third uncharacterized yeast ORF (YPL063w), share 36% identity with the 200 C-terminal residues of
Nem1p. Interestingly, sequence analysis of these proteins suggested that, like Nem1p, they might also be membrane-bound. We therefore decided to test whether they might perform a function related to the one Nem1p performs at the nuclear/ER membrane biogenesis (26).

Psr1p and Psr2p are very closely related (83% identity over their conserved C-domains and 29% identity over their 175 N-terminal portion) suggesting that they perform a redundant function. Indeed, we found that the psr1psr2, but not the psr1 or psr2 mutant, exhibits a strong growth inhibition when challenged with high concentrations of sodium chloride. Other aspects of the cell biology of the psr1psr2 cells, like cell wall and plasma membrane structure, actin organization, or endocytosis, are not compromised. Accordingly, the observed defect seems to be due to a specific block of a stress response pathway that is required for survival under sodium ion stress. Indeed, we found that upon increase of sodium concentration, the psr1psr2 mutant does not induce properly expression of PMR2/ENA1, the major sodium extrusion pump of yeast cells. Yeast cells are very sensitive to even minor reduction of Ena1p/Pmr2p levels and mutations that reduce even close to 50% the induction of PMR2 transcription, inhibit growth in cells undergoing sodium stress (7, 12).
How do Psr1p/Psr2p induce *PMR2* expression under conditions of sodium stress? Since the *psr1psr2* mutant does not exhibit general osmotic defects, it appears that the function of Psr1p/Psr2p might be independent of the *HOG* pathway, which is responsible for the response to non-specific osmotic stress. Furthermore, whereas the *HOG*-mediated induction of *PMR2* has been reported to take place at low salt concentrations (0.3M) (7), the *psr1psr2* mutant does not display any growth defect at this concentration (our unpublished observations). On the other hand, a second HOG-independent pathway required for induction of *PMR2* has been described, which is dependent on the conserved type 2B phosphatase calcineurin (7, 11, 12). Interestingly, our data show that inactivation of calcineurin function in a *psr1psr2* mutant under conditions of sodium stress, has an additive effect, both on growth, as well as in the expression of *PMR2*. This observation indicates that Psr1p/Psr2p function in a sodium stress response pathway that is parallel and independent of the calcineurin mediated pathway.

Response to sodium stress can be modulated in several ways and can involve mechanisms which do not directly utilize the induction of *ENA1/PMR2*. For example, it has been recently demonstrated that activation of the Trk1p/Trk2p potassium transporter system can
decrease the membrane potential and therefore reduce uptake of toxic cations (32). Although we cannot exclude a similar parallel function of Psr1p/Psr2p in a distinct transport system, our data link the sodium sensitivity of the \textit{psr1psr2} mutant with the expression of \textit{ENA1/PMR2}. Accordingly, Psr1p/Psr2p may define a novel sodium stress response pathway that is involved in the activation of \textit{PMR2}. How information about sodium concentration in and out of the yeast cell is sensed at the molecular level, is not known. In bacteria, two Na\textsuperscript{+}, Li\textsuperscript{+}/H\textsuperscript{+} antiporters, NhaA and NhaB, have been characterized that are essential for adaptation in high salinity (33, 34). The expression and activity of NhaA has been shown to be modulated by changes in ion concentration, mediated by the positive regulator NhAR (35).

Interestingly, because of its distinct plasma membrane location, Psr1p (and presumably Psr2p), could be closely linked to the initial event of detecting and transmitting the ion stress signal, through the interaction with a plasma membrane sodium sensor. The two other phosphatases involved in the induction of \textit{ENA1/PMR2} expression, calcineurin and Ppz1p, carry myristoylation signals, but they do not appear to localize to the plasma membrane (36, 37).

The conserved C-terminal domain of Psr1p/Psr2p shares homology with Fcp1p, which was recently shown to function as the major
phosphatase of the carboxy-terminal domain of the large subunit of RNA polymerase II (20). Neither Psr1p/Psr2p nor any other member of this conserved protein family exhibits sequence similarity with any of the 31 predicted protein phosphatases encoded by the yeast genome (38). We demonstrate here that Psr1p has phosphatase activity against an artificial substrate (pNPP), that is dependent on the short DXDX(T/V) motif, initially identified in a family of phospho-transferases and phospho-hydrolases (19). Although we find that mutations in either aspartic acid inhibit the function of the protein in vivo, both mutant proteins, purified from yeast extracts, have a residual activity against pNPP. Similar experiments with Fcp1p showed that the first aspartic acid, acting as the phospho-acceptor site in the corresponding motif of human phosphomannomutase (19), is absolutely essential for activity against pNPP (20). This difference might reflect different properties of the Psr1p C-terminal domain as compared with Fcp1p. Indeed, such a difference is implied by sequence comparison between Psr1p/Nem1p and the other members of this family and Fcp1p (see below).

Our data show that Psr1p is targeted and directly associated with the plasma membrane via a short amino-terminal sequence that is also conserved in Psr2p. Interestingly, ER-membrane targeting of Nem1p,
the other characterized member of this family, also depends by its amino-terminal half, that contains an unusual transmembrane sequence (26). It seems therefore that during evolution, the different phosphatase domains of the Psr1p/Nem1p family have been fused to distinct targeting signals that allow them to function in different places within the cell.

The Psr1p/Nem1p family: more than a phosphatase domain?

Sequence alignment of the members of the Psr1p/Nem1p family leads to two interesting observations: (a) Fcp1p is divergent to the other members of this family. This becomes more evident in the C-terminal half of the Psr1p/Nem1p homology domain (underlined in Figure 9) where all 14 proteins from different species, except Fcp1p, exhibit a very regular pattern of identity. This suggests the presence of an additional conserved sequence of unknown function, linked to proteins containing the DXDX(T/V) motif. (b) we identified three members of this family (SPBC8D2.21C, YPL063w and T21C9.12) which, although they share significant homology with the entire Psr1/Nem1p conserved domain, lack the DXDX(T/V) phosphatase motif (Figure 9). If indeed the DXDX(T/V) does function as the unique phosphoacceptor site in all
these proteins, this would imply that the Psr1p/Nem1p domain has a very conserved function and/or folding which is independent of the phosphatase activity. Alternatively, there might be more than one phosphoacceptor sites within this domain. Interestingly, there appears to be a conserved DXXD stretch C-terminal to the DXDX(T/V) motif in all members of this family except Fcp1p.

In summary, we identified a novel pair of plasma membrane anchored phosphatases which participate in the regulation of ENA1/PMR2 expression in response to sodium stress. It will be interesting to identify the substrates of the phosphatases in vivo, and to determine the function of the other conserved stretches found within their C-terminal catalytic domains.

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LEGENDS TO THE FIGURES

Fig. 1 Primary structure of Psr1p and Psr2p
A.) Schematic representation of the primary structure of Psr1p and Psr2p. The hatched boxes within Psr1p and Psr2p represent their highly conserved carboxy-terminal domains. Black boxes indicate the putative lipid attachment sites in both proteins. Psr1p contains also a short polyglutamine stretch followed by 10 (P/S)Q repeats, upstream of its conserved C-domain.
B.) Sequence alignment of Psr1p and Psr2p. Sequences were aligned using Clustal W1.7 and displayed with the program “Boxshade” (http://www.ch.embnet.org/software/BOX_form.html).

Fig. 2 GFP-tagged Psr1p localizes at the plasma membrane.
Subcellular localization of Psr1p-GFP in live cells. PSR1-GFP was expressed from a low copy (ARS/CEN) or high copy (2μ) number plasmid transformed into the psr1::HIS3 disrupted strain.

Fig. 3 Plasma membrane binding of Psr1p is mediated by its conserved N-terminal residues
A.) Kyte-Doolittle hydrophobicity plot for Psr1p and Psr2p. The window size used is 15 amino acids.
B.) Psr1p behaves biochemically as integral membrane protein. Spheroplasts expressing a Psr1p-PtA fusion protein were lysed as described in Materials and Methods and a soluble (S100) and insoluble (P100) fraction were obtained (“buffer”). The P100 fraction was then extracted with a buffer containing 1% Triton X-100 (“TX-100”) or 1M NaCl (“NaCl”). After centrifugation, equivalent amounts of the insoluble pellet (P100) and the supernatant (S100) were analyzed by SDS-PAGE followed by Western blotting using anti-PtA or anti-Dpm1p antibodies.

C.) Two cysteine residues close to the N-terminus of Psr1p are essential for its membrane association. Upper panel: Sequence alignment of the N-terminal residues of Psr1p and Psr2p. Arrows indicate the conserved glycine and cysteine residues. Lower panel: Strains expressing either wild-type (PSR1) or the G2A (PSR1-G2A) and C9G, C10G (PSR1-C9G,C10G) mutants of Psr1p were spheroplasted and lysed in a detergent-free buffer (H; homogenate). After centrifugation, equivalent amounts of the insoluble pellet (P) and the supernatant (S) were analyzed by SDS-PAGE followed by Western blotting using anti-PtA antibodies.

D.) Targeting of Psr1p to the plasma membrane is mediated by its extreme N-terminus. A fusion protein between the first 28 Psr1p residues and GFP was expressed into the psr1psr2 mutant from a centromeric vector.
E.) Psr1p is phosphorylated within its N-terminal domain. Psr1p, either full-length (Psr1p-PtA) or lacking its C-terminal conserved domain (Psr1ΔCp-PtA) was affinity-purified on IgG-Sepharose beads and treated with 10 units of alkaline phosphatase (ALP) for 15 min at room temperature. The fusion proteins were then eluted with low pH and analyzed by SDS-PAGE followed Western blotting using anti-ProtA antibodies, as described under Materials and Methods. The bands migrating between 40 and 50 kDa are proteolytic fragments of the full-length Psr1p (see also Figure 8).

Fig. 4 Psr1p and Psr2p are required for growth under conditions of sodium ion stress

Left panel: Growth properties of the psr1, psr2 and psrlpsr2 mutants under various stress conditions. Precultures of psr1::HIS3, psr2::TRP1 and psr1::HIS3 psr2::TRP1 strains (psrlPSR2, PSR1psr2 and psrlpsr2 respectively), as well as of the isogenic wild-type strain (PSR1PSR2) were diluted in liquid YPD medium and an equivalent number of cells from four serial dilutions, were spotted onto YPD plates containing either no addition, 1M sorbitol, 1M NaCl or 1M KCl. Plates were incubated at 30°C for 2 to 5 days. Right panel: Growth properties of the hog1 and the PSR1PSR2, PSR1psr2 and psrlpsr2 mutants on YPD plates containing 1M KCl. Plates were incubated at 30°C for 3 days.
Fig.5 *PSR1* and *PSR2* are mediators of *ENA1/PMR2*

transcriptional activation under conditions of sodium ion stress

A.) Constitutive expression of *ENA1/PMR2* can suppress the growth inhibition of the *psr1psr2* cells upon sodium stress. *psr1psr2* transformants containing either two empty vectors, a URA-plasmid expressing *PMR2* under the control of a constitutive promoter (plasmid pJQ10, (25)) or the *PMR2* containing plasmid plus a vector expressing *PSR1*, were spotted onto a YPD plate containing 1M NaCl and incubated for 4 days at 30°C.

B.) *PSR1* and *PSR2* mediate induction of *PMR2* transcription upon sodium stress. The *psr1psr2* mutant (white bars) or the isogenic wild-type strain (*PSR1PSR2*-black bars) were transformed with a pmr2::LacZ reporter gene (plasmid pFR70, (7)), grown in selective media and then diluted into YPD media for 6h in the presence or absence of 1M NaCl. β-Galactosidase activity was assayed in the extracts of four independent transformants as described in Materials and Methods.
Fig. 6 The PSR1/PSR2 mediated PMR2 induction is independent of the calcineurin function

A.) PSR1/PSR2 and calcineurin function in parallel sodium stress response pathways. Precultures of psr1psr2, PSR1psr2 or PSR1PSR2 strains were diluted in liquid YPD medium and equivalent numbers of cells from three serial dilutions were spotted onto YPD plates supplemented with 0.4M NaCl, in the presence or absence of 1µg/ml FK506, as indicated.

B.) PMR2 expression is regulated independently by PSR1/PSR2 and calcineurin. The psr1psr2 mutant and the isogenic wild-type strain (PSR1PSR2) expressing a pmr2::LacZ reporter gene (plasmid pFR70, (7)), were grown in selective media and then diluted into YPD media for 6h in the presence or absence of 1M NaCl and 1µg/ml FK506, as indicated. β-Galactosidase activity was assayed in the extracts of two independent transformants as described in Materials and Methods.

Fig. 7 Psr1p contains an essential DXDX(T/V) phosphatase motif

The psr1psr2 mutant was transformed with YEplac181-LEU2, either empty or expressing the wild-type PSR1-PtA fusion, the PSR1ΔC-PtA truncation mutant, or the point mutants in the DXDX(T/V) motif of PSR1, (PSR1D(263)E-PtA and PSR1D(265)E-PtA). As indicated, all alleles
were tagged C-terminally with two IgG-binding domains from Protein A. Growth of the isogenic wild-type strain was also followed. The corresponding transformants were spotted onto YPD plates containing 1M NaCl and incubated for 4 days at 30°C.

**Fig. 8** Psr1p has phosphatase activity *in vitro*

A.) Affinity purification of Psr1p-PtA fusions from yeast extracts. Protein A fusions of either wild-type Psr1p (Psr1p-PtA), or different Psr1p mutants (Psr1ΔCp-PtA, Psr1(D263E)p-PtA and Psr1(D265E)p-PtA), and a control consisting only of PtA expressed under the control of theNOP1 promoter, were expressed and affinity purified from the psr1psr2 mutant by IgG-Sepharose chromatography as described in Materials and Methods. The purified proteins were analyzed by SDS-PAGE and Coomassie staining (top panel) or Western blotting (lower panel) using anti-PtA antibodies. The position of molecular weight markers (in kDa) is indicated.

B.) Psr1p-PtA can hydrolyze p-nitrophenylphosphate (pNPP). IgG-Sepharose beads carrying the PtA fusions from (A) were incubated with 200µl of pNPP for 30 min at 30°C as described in Materials and Methods. Absorbance of the generated p-nitrophenol (pNP) was measured at 410 nm. The PtA control sample was used as a reference. Approximately 10µg from each fusion were used in the assay (3x the amount shown in
the Coomassie gel in A). One µg Psr1p-PtA generates 1 nmol p-nitrophenol per 30 min. Each PtA-fusion was assayed four times and the SD is representing the error between these samples.

**Fig. 9 The function of the conserved C-domain of the Psr1p/Nem1p family may not be restricted to DXDX(T/V) motif-dependent phosphatase activity**

Sequence alignment of proteins found in the databases that show homology over the conserved C-domains of Psr1p and Nem1p:

*S. cerevisiae* Psr1p (residues 235-427), Nem1p (residues 231-446), Fcp1p (residues 160-374) and YPL063w (residues 173-361),

*C. elegans* F45E12.1 (residues 45-246), T21C9.12, *D. discoideum* AF111941.1 (residues 121-306) *H. Sapiens* HYA-22 (residues 153-340), OS4 (79-283), CAA09865.1 (residues 45-244) *S. pombe* SPBC3B8.10C (residues 246-476), SPBC8D2.21C (residues 158-346), YA-22 (residues 121-325). Sequences were aligned using Clustal W1.7 and displayed using SeqVu 1.0. Only identities among different sequences are highlighted. Both the DXDX(T/V) motif (thick line) and the C-terminal portion of the homology domain (thin line) are underlined.
### Table I. Yeast strains used in this study

| Strain                  | Relevant genotype                                                                 | Ref. or source                  |
|-------------------------|----------------------------------------------------------------------------------|--------------------------------|
| RS453                   | *Mata/α ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3*                        | R. Serrano                     |
| *psr1*                  | *Mata ade2 his3 leu2 trp1 ura3 psr1::HIS3*                                        | This study                     |
| *psr2*                  | *Mata ade2 his3 leu2 trp1 ura3 psr2::TRP1*                                        | This study                     |
| *psr1psr2*              | *Mata ade2 his3 leu2 trp1 ura3 psr1::HIS3 psr2::TRP1*                             | This study                     |
| *PSR1-GFP*              | *Mata ade2 his3 leu2 trp1 ura3 psr1::HIS3*                                       | This study                     |
| *PSR1-PtA (CEN)*        | *Mata ade2 his3 leu2 trp1 ura3 psr1::HIS3 (YCplac111-LEU2-PSR1-PtA)*             | This study                     |
| *PSR1-PtA (2µ)*         | *Mata ade2 his3 leu2 trp1 ura3 psr1::HIS3 psr2::TRP1 (YEplac181-LEU2-PSR1-PtA)* | This study                     |
| *PSR1(D263E)-PtA (2µ)*  | *Mata ade2 his3 leu2 trp1 ura3 psr1::HIS3 psr2::TRP1 (YCplac181-LEU2-PSR1(D263E)-PtA)* | This study                     |
| *PSR1(D265E)-PtA (2µ)*  | *Mata ade2 his3 leu2 trp1 ura3 psr1::HIS3 psr2::TRP1 (YCplac181-LEU2-PSR1(D265E)-PtA)* | This study                     |
| *PSR1ΔC-PtA (2µ)*       | *Mata ade2 his3 leu2 trp1 ura3 psr1::HIS3 psr2::TRP1 (YCplac181-LEU2-PSR1ΔC-PtA)* | This study                     |
Fig. 2

**PSR1-GFP**

- low-copy
- high-copy
Fig. 3

A

\(\text{Psr1p}\)

\(\text{Psr2p}\)

-3
-2
-1
0
1
2
3

100
200
300
400

B

buffer
TX-100
NaCl

|    | Extract | S100 | P100 | S100 | P100 |
|----|---------|------|------|------|------|
| Psr1p |    |    |      |      |      |
| Psr2p |    |    |      |      |      |

C

\(\text{Psr1p}\)

\(\text{Psr2p}\)

\(\text{PSR1}\)

\(\text{PSR1-G2A}\)

\(\text{PSR1-C9G,C10G}\)

\(\alpha\text{-ProtA}\)

D

\(\text{Psr1p}\Delta(S28)-GFP}\)

E

\(\text{ALP}\)

\(\alpha\text{-ProtA}\)

- 70
- 60
- 50
- 40
- 30
- 20
- 10
- 0

\(\text{Psr1p-PtA}\)

\(\text{Psr1ΔCp-PtA}\)
Fig. 5

A

B

psr1 psr2

+ pURA
pLEU

+ pURA-PMR2
pLEU

+ pURA-PMR2
pLEU-PSR1

1M NaCl

β-galactosidase units

pmr2::LacZ

- NaCl

+ NaCl

PSR1 PSR2

psr1 psr2
**Fig. 6**

**A**

- PSR1 PSR2
- psr1 psr2
- PSR1 psr2

- 0.4M NaCl
- 0.4M NaCl + FK 506

**B**

`pmr2::LacZ`

|          | PSR1 PSR2 | psr1 psr2 |
|----------|-----------|-----------|
| 1M NaCl  | -         | +         |
| FK506    | -         | +         |

**β-galactosidase units**

- 0.4M NaCl
- 0.4M NaCl + FK 506

- 1M NaCl
- 1M NaCl + FK 506
Fig. 7

| Growth in 1M NaCl | Strain         |
|-------------------|---------------|
|                   | wild-type     |
|                   | + vector      |
|                   | + PSR1-PtA    |
|                   | + PSR1ΔC-PtA  |
|                   | + PSR1(D263E)-PtA |
|                   | + PSR1(D265E)-PtA |

|                  | Strain          |
|------------------|-----------------|
| PSR1 PSR2        |                 |
| psr1 psr2        |                 |
Fig. 8

A

B

$A_{(410)}$ nmol pNP
Fig. 9
Psrlp/Psr2p, two plasma membrane phosphatases with an essential DXDX(T/V) motif required for sodium stress response in yeast

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