Functional Characterization of the Yeast Ppz1 Phosphatase Inhibitory Subunit Hal3

A MUTAGENESIS STUDY

Received for publication, May 20, 2004, and in revised form, July 28, 2004
Published, JBC Papers in Press, July 29, 2004, DOI 10.1074/jbc.M405656200

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Saccharomyces cerevisiae Hal3 is a conserved protein that binds the carboxyl-terminal catalytic domain of the PP1c (protein phosphatase 1)-related phosphatase Ppz1 and potently inhibits its activity, thus modulating all of the characterized functions so far of the phosphatase. It is unknown how Hal3 binds to Ppz1 and inhibits its activity. Although it contains a putative protein phosphatase 1c binding-like sequence (KKHLVLFXX), no mutagenesis analysis suggests that this motif is not required for Ppz1 binding and inhibition. The mutation of the conserved His778 (possibly involved in dehydrogenase catalytic activity) did not impair Hal3 functions or Ppz1 binding. Random mutagenesis of the 228 residue-conserved central region of Hal3 followed by a loss-of-function screen allowed the identification of nine residues important for Ppz1-related Hal3 functions. Seven of these residues cluster in a relatively small region spanning from amino acid 446 to 480. Several mutations affected Ppz1 binding and inhibition in vitro, whereas changes in Glu460 and Val462 did not alter binding but resulted in Hal3 versions unable to inhibit the phosphatase. Therefore, there are independent Hal3 structural elements required for Ppz1 binding and inhibition. S. cerevisiae encodes a protein (Vhs3) structurally related to Hal3. Recent evidence suggests that both mutations are synthetically lethal. Surprisingly, versions of Hal3 carrying mutations that strongly affected Ppz1 binding or inhibitory capacity were able to complement lethality. In contrast, the mutation of His778 did not. This finding suggests that Hal3 may have both Ppz1-dependent and independent functions involving different structural elements.

In contrast with the high number of protein kinases, eukaryotic cells contain a relatively small number of proteins with Ser/Thr protein phosphatase activity. A general strategy to refine specificity and to allow fine control of thousands of different dephosphorylation reactions in the cells has been the association of a catalytic subunit with a number of different regulatory subunits, which would confer to the catalytic protein specificity for given substrate(s) or restricted subcellular location. Such a strategy is particularly important in the case of type I Ser/Thr phosphatases for review see Refs. 1 and 2).

Ppz1 is a type 1-related yeast Ser/Thr protein phosphatase composed of a catalytic carboxyl-terminal domain and an NH2-terminal extension (3). The catalytic domain is approximately 60% identical to the mammalian and plant catalytic subunits of protein phosphatase 1 (PP1c).1 In yeast cells, Ppz1 is involved in a variety of cell processes including regulation of salt tolerance, maintenance of cell wall integrity, and regulation of cell cycle at the G1/S transition (4). These findings can be explained as a result of an inhibitory activity on the Trk1/Trk2 potassium transporters (5), although Trk-independent functions have been also reported (6).

The activity of Ppz1 is regulated by Hal3/Sis2, a conserved protein identified several years ago in two independent screens as a high-copy suppressor of the sit4 growth defect (7) and by its capacity to confer halotolerance (8). Hal3 acts as a negative regulatory subunit of Ppz1 by binding to the phosphatase carboxyl-terminal catalytic domain (9) and strongly inhibits Ppz1 activity, thus modulating its diverse physiological functions. Therefore, the overexpression of Hal3 provides increased salt tolerance, whereas hal3 cells are hypersensitive to sodium and lithium cations. Sit4 and hal3 mutations display synthetic lethality due to G1 blockade, whereas high-copy expression of HAL3 accelerates entry into S phase after an α-factor-induced G1 arrest in a sit4 mutant (7, 10). Finally, high-copy expression of Hal3 aggravates the lytic phenotype of a Slt2/Mpk1 MAP kinase mutant, whereas, in contrast, a lack of HAL3 improves growth of this strain (9).

Homologs of Hal3 have been found in plants and animals (11), although they lack the acidic tail. The Arabidopsis thaliana AtHal3a isoform was found to be a flavoprotein able to partially complement a hal3 yeast mutant. Resolution of its three-dimensional structure allows Albert et al. (12) to propose that the plant protein could act as a dehydrogenase through a mechanism that might involve His778, a residue conserved in yeast Hal3 (His778). Further work uncovered that AtHal3a

1 The abbreviations used are: PP1c, protein phosphatase 1; CM, complete minimal medium; CM-uracil, CM lacking uracil; GST, glutathione S-transferase.
could catalyze the decarboxylation of 4′-phosphopantothenoylcysteine (13), suggesting that this protein could be involved in coenzyme A biosynthesis, and pointed out an important role of Cys\textsuperscript{755} in the catalytic mechanism (13, 14). It is worth noting that a such Cys residue is not conserved in yeast Hal3. The *S. cerevisiae* genome contains two genes, VHS3 and YKL088w, encoding proteins that are 49 and 28\% identical to Hal3, respectively. Recent evidence suggests that Vhs3 could be functionally related to Hal3 on the basis that high-copy VHS3 partially complements the absence of Hal3 function (15) and that recent work in our laboratory has revealed that Vhs3 binds to and inhibits Ppz1 in vitro (16).

The mechanism of binding between PP1c and its different regulatory subunits and how these interactions modulate the phosphatase activity has received considerable attention in the last few years, particularly after the elucidation of the PP1c structure (17, 18). A number of structural features shared by most known PP1c regulatory subunits have been defined. The most common one is the existence of a motif, initially identified as a RVXF sequence (19, 20) and subsequently found in many regulatory subunits as more or less conserved variations of the original sequence. In addition to this motif, additional interaction sites are probably required in many cases (for review see Ref. 1). In contrast, besides the early observation that the characteristic highly acidic tail of Hal3 is required for function in vivo (7, 8), very little is known regarding the structural elements defining the cellular role of Hal3 or its function as phosphatase inhibitor. Interestingly, Hal3 does not structurally resemble previously characterized PP1c phosphatase inhibitors and it does not bind or inhibit in vitro yeast PP1c (encoded by GLC7 in *S. cerevisiae*) (9, 21). Therefore, previous experience and knowledge on the regulation of Glc7 by its inhibitors and it does not bind or inhibit yeast PP1c

**MATERIALS AND METHODS**

**Growth Conditions of *Escherichia coli* and *S. cerevisiae* Strains—** Except when otherwise indicated, *E. coli* DH5\textalpha strain was used as plasmid DNA host and was grown in LB medium at 37 °C supplemented with 50 μg/ml ampicillin when needed for plasmid selection. Yeast cells were grown unless otherwise stated at 28 °C in YPD medium or in complete minimal medium (CM) lacking the appropriate requirements for plasmid selection. All of the yeast strains used in this work are derived from JA-100 (9) and are listed in Table 1.

**Recombinant DNA Techniques and Plasmid Construction—** *E. coli* and *S. cerevisiae* cells were transformed using standard techniques as previously described (9). Restriction reactions, DNA ligations, and other standard recombinant DNA techniques were carried out as described previously (22).

**Sources for the HAL3 gene was plasmid YEp351-HAL3, which contains a 2.4-kbp Bell-HindIII DNA genomic fragment (8). Plasmid pGEM3Z-HAL3 was constructed by digestion of YEp351-HAL3 with EcoRI/HindIII and cloning of the 2.4-kbp fragment into the same sites of plasmid pGEM3Z (Promega). Plasmid YEplac195-Hal3 was obtained by cloning the EcoRI/HindIII/HAl3 fragment into the same sites of YEplac195 (23).

**Mutation of His\textsuperscript{375} to glycine was made by PCR using oligonucleotides 5′-Hal3H265AR and 3′-Hal3H1paf (see Supplemental Table) to amplify a fragment of ~700 bp that was then digested and cloned into the BamHI/Hpal sites of the gapped YEplac195-Hal3 to yield YEplac195-Hal3(H265G).**

**Random PCR Mutagenesis and Screen for Loss of Hal3 Activity—** Random PCR mutagenesis was performed essentially as described by Fromant et al. (24) using MscI\textalpha at a final concentration of 4.7 mm to minimize the occurrence of insertions and/or deletions. YEps51-HAL3 and oligonucleotides 5′-Hal3F268A and 3′-Hal3Hpal were used to amplify the 716-bp fragment between the BamHI and the Hpal sites found in the HAL3 coding sequence. Four different reactions were made in the presence of one of the forcing dNTPs. The products of several independent PCR reactions were pooled, purified, and digested with BamHI and Hpal and cloned in the same sites of the gapped plasmid pGEM3Z-HAL3. Ligation products were introduced into *E. coli* competent cells by electroporation. Approximately 30,000 independent colonies were recovered and mixed. Plasmid DNA was prepared, digested with EcoRI/HindIII, and electrophoresed. The 2.4-kbp band was recovered and cloned into YEplac195 to yield at least 30,000 colonies.

**The plasmid library was used to transform strain JC010 (slt2Δ) using enough DNA to yield around 3000 transformants/plate (determined using control CM lacking uracil (CM-ura) medium, and plates were incubated for 48–72 h.** Clones able to generate macroscopic colonies under these conditions (usually 20–30/plate) were picked out and grown for an additional 3–6 h in sterile 96-well plates filled with CM-ura medium. They were then replicated in CM-ura plates and in the same plates containing 1 m sorbitol. Approximately 40,000 transformants were plated in CM-ura-cil medium, and plates were incubated for 48–72 h. Clones able to generate macroscopic colonies under these conditions (usually 20–30/plate) were picked out and grown for an additional 3–6 h in sterile 96-well plates filled with CM-ura-cil medium. They were then replicated in CM-ura-cil plates and in the same plates containing 1 m sorbitol, 1 m NaCl, 9.2 m LiCl, or 3 m caffeine for initial characterization of the clones. Plasmids from the selected clones were then extracted, amplified in *E. coli*, and subjected to restriction mapping to verify the nature of the insert. The constructs were reintroduced in strain JC010, and the phenotypes were reassessed as indicated above. Clones showing a consistent behavior were considered positives and subjected to further analysis. To ensure that the absence of function did not result from truncations of the protein, the presence of the entire Hal3 protein was assessed. For this purpose, yeast cell lysates of selected clones were prepared as described by de Nadal et al. (9, 40 μg of total protein were analyzed by SDS-PAGE, and Hal3 was immunodetected using anti-Hal3 polyclonal antibodies. The plasmids expressing a full-length protein were subjected to sequence analysis to confirm the entire *BamHI/-Hpal fragment in search of mutations producing a change in the amino acid sequence of the protein that could be responsible for the loss of Hal3 function.

**All of the mutated versions of HAL3 were removed from YEplac195 by digestion with EcoRI and HindIII, and the resulting 2.4-kbp frag-
ments were cloned into the same sites of plasmids YCp33 (URA3 marker), YCp22 (TRP1 marker), and YEpplac195 (TRP1 marker).

**In Vitro Binding Assays**—In vitro binding assays were performed as follows. GST-Ppz1Δ344 was expressed in bacteria and bound to glutathione-agarose beads essentially as described previously (21). Yeast extracts of strain IM021 (ppz1 hal3) transformed with the YEpplac195 multicopy plasmids carrying the different versions of Hal3 under study were basically prepared as described by de Nadal et al. (9). 1 mg of total protein from each sample was mixed with 50 μl of the affinity beads and incubated for 1 h at 4 °C with gentle shaking. The washing procedure was essentially as described previously (9) with the exception that the beads were finally resuspended in 100 μl of 2× SDS sample buffer and boiled. Samples (10 μl) were analyzed by SDS-PAGE and probed using anti-Hal3 polyclonal antibodies.

**In Vitro Phosphatase Assays**—The effect of the different versions of Hal3 as inhibitors of Ppz1 phosphatase activity was analyzed using bacterially expressed proteins. To this end, the entire open reading frame of the different HAL3 versions was amplified by PCR using oligonucleotides 5′-HAL3ECorH and 3′-HAL3 Xhol to generate a 1.7-kbp fragment that was then cloned into these same sites of plasmid pGEX6P-1 (Amersham Biosciences). The different versions of GST-Hal3 generated were expressed using 1 mM isopropyl-β-D-thiogalactoside for induction at 37 °C for 3 h. Conditions of expression and purification of bacterial recombinant GST-Ppz1Δ344 were essentially as described previously (21). Once bound to the glutathione-agarose affinity column, the recombinant phosphatase was treated for 4 h at 4 °C with PreScission protease (Amersham Biosciences) following the manufacturer’s indications (80 units/ml resin) to cleave the GST moiety. The eluted GST-free phosphatase was analyzed by SDS-PAGE and quantified.

Because bacterial expression of the different GST-Hal3 versions produced variable amounts of shorter polypeptides, the amount of intact GST-Hal3 present in the samples used in the assays was determined as follows. A 10-μl aliquot of each version eluted from the glutathione-agarose affinity column was analyzed by SDS-PAGE and Coomassie Blue-stained. The gel was scanned, and the amount of intact protein in each sample was quantified by comparison using commercial software with different amounts of a bovine serum albumin solution of known concentration.

The Ppz1 phosphatase activity was measured using p-nitrophenylphosphate as substrate essentially as described previously (21) with the following modifications. 0.5 μg of Ppz1 phosphatase was used, the concentration of substrate was 10 mM, and the assay was carried out for 20 min at 30 °C. Different amounts of each version of GST-Hal3 were incubated in the presence of the phosphatase for 5 min at 30 °C, and the assay was started by the addition of the substrate.

**Other Techniques**—Growth on plates (drop tests) was assessed as described previously (25). Random spore analysis was performed essentially as described previously (26).

**RESULTS**

**Evaluation of the Functional Relevance of His<sup>378</sup> and the 265<sup>HVLF</sup>268 Residues in Hal3**—As mentioned in the Introduction, Hal3 does not exhibit significant similarity with known regulatory subunits of type 1 protein phosphatases. However, it contains a HVLF sequence (residues 265–268) that resembles the consensus RVXF sequence found in many PP1c regulatory subunits and we considered it necessary to evaluate whether this region could act as a regulatory element. To this end, we mutated His<sup>378</sup> to glycine and Phe<sup>268</sup> to alanine and tested the ability of these versions of Hal3 to mimic the function of the native protein, both at normal levels and under overexpression conditions. As shown in Fig. 1, the mutation of Phe<sup>268</sup> to Ala did not affect the ability of the protein to restore wild type tolerance in a hal3 strain, whereas a change of His<sup>378</sup> to Gly reduced this ability partially (Fig. 1, upper panel). When these HAL3 versions were overexpressed, they behaved essentially as the wild type forms (data not shown). When overexpressed, both versions were also able to block growth of a slt2Δmpk1 strain in the absence of sorbitol, similarly to native Hal3 (Fig. 1, middle panel). Finally, when they were expressed in low-copy (Fig. 1, lower panel), both versions allowed the growth of strain JC002 (slt4 tetO:HAL3), which suffers a G<sub>1</sub>S blockade in the presence of doxycycline due to lack of HAL3 expression (10).

On the basis of the structure of the AtHal3a protein, it was reasonable to assume that His<sup>378</sup> in *S. cerevisiae* Hal3 (equivalent to AtHal3a His<sup>360</sup>) could have functional relevance. However, when we mutated this residue to Ala and performed all of the functional tests described above, this mutated version of Hal3 displayed a completely wild type phenotype (Fig. 1). Previous work has shown that the Hal3 functions tested above are mediated by Ppz1 and result from inhibition of this phosphatase. Therefore, we investigated whether these mutated versions were still able to effectively bind and inhibit Ppz1. A GST-fused version of the phosphatase domain was expressed in *E. coli* and bound to glutathione-agarose to form an affinity system for Hal3. The different Hal3 versions were expressed at similar levels in *ppz1* yeast cells, and extracts were allowed to bind to the affinity beads. As shown in Fig. 2, left panel, the F268A and H378A versions were able to bind Ppz1 as the wild type form, whereas the H265G form was slightly less effective. To test the capacity of the different Hal3 versions to inhibit the catalytic activity of Ppz1, they were expressed in *E. coli* and in vitro inhibition assays were carried out. As it can be observed in Fig. 2, right panel, the F268A and H378A versions displayed an inhibitory potency very similar to that of wild type Hal3. The H265G mutated form allowed almost full inhibition of Ppz1, although it was slightly less effective than native Hal3.

In conclusion, these results indicate that the 265<sup>HVLF</sup>268 motif in Hal3 is probably not critical for Ppz1 binding and inhibition. Similarly His<sup>378</sup>, although theoretically equivalent to AtHal3a His<sup>360</sup>, is not relevant for Ppz1 regulation.

**A Screen for Mutations Resulting in Loss of Function Reveals a Small Region in Hal3 Important for Ppz1 Binding and/or Inhibition**—As the approach described above failed to reveal residues important for regulation of Ppz1 by Hal3, we decided...
to create a PCR-based library of mutated versions of the regulatory protein and set up a screen for loss of function. The region was subjected to mutagenesis encompassed from Arg506 to Ile586 just upstream of the highly acidic tail, and it corresponds to the most conserved region among eukaryotes. This approach depicted in Fig. 3 is based on the ability of high-copy expression of HAL3 to inhibit growth in synthetic medium of a slt2/mpk1 mutant except if an osmotic stabilizer, such as sorbitol, is present. Therefore, transformants growing in the non-permissive conditions should harbor non-functional forms of Hal3. The screen of approx 40 000 colonies yielded around 225 positive clones. The subsequent analysis of the plasmid insert allowed the discarding of ~50% of those inserts. The remaining clones were introduced again in strain JC10 (slt2/mpk1) and re-tested for growth. Protein extracts were prepared from 85 clones, subjected to SDS-PAGE electrophoresis, and transferred to membranes, and immunoblots were developed using anti-Hal3 polyclonal antibody. Approximately 80% of the clones did not give signal with the antibody, did not contain a full size protein (probably due to premature stop codons), or exhibited the right protein size but at low levels of expression and therefore were also discarded. 17 clones passed all of these tests and were subjected to DNA sequencing in search of mutations. The relevant changes identified in this study are shown in Table II. Nine changes appeared to be unique. Interestingly, they were not scattered through the entire region that was subjected to mutagenesis but mostly concentrated in the last 40 carboxy-terminal residues. In three cases, multiple mutations affecting more than one amino acid were found. It is worth noting that two of them included a change also found as a single mutation. The third one represents a triple change in which one of the mutations (S459P) lies in the vicinity of several residues affected by single mutations. These multiple mutated versions have not been further characterized.

As it can be deduced from Fig. 3, left panel, the potency of the phenotype exhibited by the different Hal3 versions was not identical when they were tested in high-copy under different conditions in a slt2/mpk1 background. The mutation of Tyr313 and Ile486 resulted in a relatively weak loss of function, whereas changes affecting residues Glu460, Val462, and Asn478 allowed a vigorous growth comparable, if not stronger, to that of cells transformed with an empty plasmid. The introduction of the diverse forms of Hal3 both as a low-copy and high-copy number into hal3 cells allowed us to test the potency of the mutations in the salt tolerance phenotype. As shown in Fig. 4, the ability to confer a salt-tolerant phenotype of Hal3 versions containing changes in Tyr313 and Ile486 was somewhat less pronounced than that of the wild type protein. The mutation in Val462, Ile446, Asn466, and Asn478 resulted in a marked loss of function when present in low-copy number. However, an increase in salt tolerance could be observed when these versions were expressed in a high-copy number, suggesting that they still retained some functional capacity. In contrast, the changes in Trp452, Glu460, and Val462 resulted in a complete loss of the ability to increase tolerance to sodium or lithium ions, even when these versions were overexpressed. This pattern was reproduced quite closely when the different forms of Hal3 were introduced both in low- and high-copy numbers in strain JC002, and these cells were grown in the presence of doxycycline (Fig. 4, lower panel). In this case, the Glu460 mutation resulted in a complete loss of function.

In an attempt to explain the molecular basis for such behavior, we carried out binding experiments similar to those described above. As shown in Fig. 5, the mutation of residues Val390, Ile446, and Trp452 provoked a substantial decrease in the ability of Hal3 to bind Ppz1. Changes in Tyr313 and Ile486 probably result in a decreased binding capacity, although it is much less evident. The rest of mutations do not seem to significantly alter Hal3 and Ppz1 interaction in vitro. When the capacity of the bacterially expressed mutated forms to inhibit Ppz1 was tested, the forms that had a markedly reduced binding to Ppz1 were, as expected, unable to inhibit its phosphatase activity. Interestingly, the version Val462 despite its capacity to bind Ppz1 similarly to wild type Hal3 was less effective in inhibiting the phosphatase. The case for mutation Glu460 was remarkable as this version acted as an activator of the phosphatase. All of these results indicate that Hal3 contains residues specific for Ppz1 binding as well as residues specific for Ppz1 inhibition and that the loss of function provoked by mutations in Glu460 and Val462 results from its inability to inhibit Ppz1 while retaining the capacity to bind the phosphatase.

**Further Analysis of Residues Important for Ppz1 Binding and/or Inhibition Reveals Possible Ppz1-independent Functions for Hal3**—As mentioned in the Introduction, the *S. cerevisiae* genome contains a gene (VHS3) structurally related to HAL3 and recent work from our laboratory has suggested that the encoded protein could provide some Hal3 activity (15). We also observed recently that, after tetrad analysis or random spore analysis, it was not possible to recover viable hal3 vhs3 mutants. Furthermore, plasmid eviction experiments on plates...
containing 5-fluororotic acid showed that the \textit{HAL3} plasmid-born copy could not be lost in these cells (16), indicating again that both mutations were synthetically lethal. We considered interesting the ability to test for several of the mutations described above if they could also compromise the ability of \textit{Hal3} to complement the chromosomal mutation in a \textit{vhs3} background. To this end, we selected the His378 change as well as those mutations within the 446–480 region having the strongest effect on function according to the tests described above (Ile446, Trp452, Glu460, Val462, and Asn478). The diploid strain MAR6, heterozygotic for the \textit{hal3} and \textit{vhs3} mutations, was transformed with the mentioned versions of \textit{HAL3} as well as with the wild type gene and cloned in a centromeric plasmid, and sporulation was induced. As expected, transformation with wild type \textit{HAL3} allowed growth of all four spores in each tetrad. In contrast, in the case of the version carrying the mutation in His378, the distribution of the 27 tetrads analyzed was as follows: 4 non-parental ditype (with two growing colonies); 5 parental ditype (all four colonies grew), and 18 tetra-type (three growing colonies). Thus, we could not recover any spore containing the double mutation plus the plasmid. A similar distribution (4:4:17) was observed when 25 tetrads of the diploid strain containing an empty plasmid were analyzed. Large scale random spore analysis of the diploid containing the H378A version also failed to produce any colony exhibiting the markers associated to the mutations plus the plasmid-born marker gene. These experiments confirm our observation in that the \textit{HAL3} and \textit{VHS3} mutations present synthetic lethality (16) and suggest that the mutation of His378 affects some function of \textit{Hal3}, which is required for viability in a \textit{vhs3} background.

The diploid strain carrying any of the five selected mutations within the \textit{HAL3} 446–480 region was subjected to random spore analysis. Remarkably, in all of the cases we recovered colonies containing the three markers (between 15 and 24% of the total number of spores analyzed), which corresponded to haploid cells in >90% of the cases. In fact, these results were very similar to those obtained in control experiments in which wild type \textit{HAL3} was used. As shown in Fig. 6, these cells grew normally in both synthetic and rich media, indicating that the mutations did not affect the ability of the plasmid-born \textit{Hal3} version to allow survival of a \textit{hal3 \textit{vhs3}} strain. However, when these cells were tested for their tolerance to saline stress, the mutated \textit{HAL3} versions still displayed the loss-of-function phenotype described in a \textit{hal3} background (compare with Fig. 4). These experiments indicate that \textit{Hal3} mutations able to aboli-
is Ppz1 binding or inhibitory capacity does not affect Hal3 functions required in the absence of the VHS3 gene.

**DISCUSSION**

The biological roles of Hal3 as regulatory subunit of the Ppz1 protein phosphatase have been characterized with some detail in the last few years. However, with the exception of the existing evidence that Hal3 binds to the catalytic moiety of Ppz1 even more efficiently than to the entire protein (9), suggesting that the amino-terminal half of Ppz1 might play a protective function against inhibition by Hal3, the molecular basis on how this regulatory subunit can bind and inhibit the phosphatase was unknown. Computer analysis of the Hal3 sequence revealed a [R/K][I/V/I/X]/[F/W] motif, which resembles the consensus sequence [R/K][I/V/I/X]/[F/W] defined in many PP1c regulatory subunits (1). In yeast, similar sequences have been identified as relevant for function in several Glc7 regulatory subunits, such as Gac1 (27) or Reg1 (28), providing evidence that the Phe in this motif appears to be crucial for function. Therefore, the existing evidence supported the hypothesis that the Hal3 [R/K][I/V/I/X]/[F/W] motif could be relevant for Ppz1 interaction. However, we show that mutation of Phe [R/K][I/V/I/X]/[F/W] does not alter the functional properties of Hal3 when tested in vivo or significantly affects its capacity to bind and inhibit Ppz1. We find that the change of His [R/K][I/V/I/X]/[F/W] to glycine has some effect on the properties of Hal3, although this is probably due to the drastic modification of the size of the amino acid in this specific position. Therefore, our data do not support the idea that Hal3 and Ppz1 interact through existing Hal3 [R/K][I/V/I/X]/[F/W] motif.

Animals and plants contain proteins related to *S. cerevisiae* Hal3, although they are smaller and lack amino-terminal sequences and the carboxyl-terminal highly acidic tail. The three-dimensional structure of the AtHal3a isoform from *A. thaliana* was solved a few years ago (12), and the protein appears to be a flavoprotein whose structural features allow us to hypothesize that it could catalyze the α,β-dehydrogenation of peptidyl-cysteine and point out His [R/K][I/V/I/X]/[F/W] as a residue potentially important in the reaction. The fact that this His is conserved in *S. cerevisiae* Hal3 (His [R/K][I/V/I/X]/[F/W]) prompted us to determine whether it was relevant for its cellular function and for interaction with Ppz1. However, our data clearly show that a mutated form of Hal3 lacking His [R/K][I/V/I/X]/[F/W] is indistinguishable from the wild type version in the different test performed and, therefore, it is not relevant for Ppz1 regulation.

Because of the failure to identify relevant regulatory elements in Hal3 by comparison with known PP1c regulatory subunits, we decided to undertake a more direct approach based on a loss-of-function screen of a library of mutagenized Hal3. The region subjected to mutagenesis expanded from Tyr [R/K][I/V/I/X]/[F/W] to Arg [R/K][I/V/I/X]/[F/W], which does not include the acidic tail and corresponds to the region highly conserved between *A. thaliana* and budding yeast Hal3 proteins. It must be noted that we had to establish a control step to check that the expressed versions of Hal3 were full-length proteins, because it was reported that the acidic terminal tail was required for Hal3 function related to halotolerance and cell cycle regulation (7, 8). In fact, our screen uncovered a large number of Hal3 clones unable to provide function that, when sequenced, presented premature stop codons, thus confirming earlier data.

The screen performed on strain JC010 (slt2/mpk1) revealed nine residues expanding from Tyr [R/K][I/V/I/X]/[F/W] to Ile [R/K][I/V/I/X]/[F/W] that were relevant for function. Interestingly, the more drastic effects corresponded to changes between Ile [R/K][I/V/I/X]/[F/W] and Asn [R/K][I/V/I/X]/[F/W], i.e., a relatively small region in the vicinity of the acidic tail. A comparison of the functional incidence of these mutations under different phenotypic tests was remarkably consistent, indicating that these effects were mediated through a common mechanism. However, when the effect of these mutations on Ppz1 binding and inhibitory activity was tested, the results were not identical. Some mutations affected binding, and the strongest effects clustered in residues Val [R/K][I/V/I/X]/[F/W], Ile [R/K][I/V/I/X]/[F/W], and Trp [R/K][I/V/I/X]/[F/W]. As expected, these mutations also abolished the ability of Hal3 to inhibit Ppz1 *in vitro*. In contrast, mutations affecting Glu [R/K][I/V/I/X]/[F/W] and
Val462 did not affect Ppz1 binding but resulted in Hal3 proteins fully (Glu460) or partially (Val 462) unable to inhibit Ppz1. It is worth noting the remarkable phenotypes observed for the E460G version. Not only was it unable to complement the absence of wild type Hal3, even in high-copy number, but in some cases it appeared to aggravate the phenotype (see Figs. 3 and 4). A possible explanation for this observation would be that the expression of the E460G Hal3 version may in fact result in increased Ppz1 (and perhaps Ppz2) activity, because although being able to bind endogenous Ppz1 and therefore to displace endogenous Hal3 or the related Vhs3 protein, such binding would not result in effective inhibition of phosphatase.

**Fig. 7.** A, alignment comparing the sequence of AtHal3 and the core domain of yeast Hal3. Residues that are identical are highlighted with a light gray mask. The point mutations are indicated below the Hal3 sequence. Directed mutations are indicated with an asterisk. The predicted secondary structural elements of S. cerevisiae Hal3 are also shown. Point mutations not affecting in vitro binding to Ppz1 are highlighted with a dark gray mask. B, a ribbon (34) representation of the modeled structure of the core domain of yeast Hal3. Residues from 446 to 452, from 460 to 466, and from 478 to 480 are displayed as a black ribbon. The point mutations are labeled and indicated with arrows in a zoomed area.
activity. Alternatively, our in vitro phosphatase assays support the possibility that interaction of this Hal3 version with Ppz1 might directly result in increased catalytic phosphatase activity. In any case, our results clearly show that the Hal3 structural elements required for Ppz1 binding and inhibition cannot be independent. This finding allows the understanding of our recently reported observation that the Gdc7 inhibitor Ypi1 was able to strongly bind Ppz1 (in this case through a degenerated RVXW-like sequence) with almost negligible effect on its phosphatase activity (21).

It is remarkable that, while the mutagenesis procedure was carried out on a large part of the protein (>220 amino acids), most Hal3 residues necessary for binding and inhibition were restricted to a relatively small region (residues 446–480), indicating that this region has a key role in Hal3 functions involving modulation of Ppz1. Although the three-dimensional structure of S. cerevisiae Hal3 has not been solved, modeling calculations for the yeast Hal3 256–480 region against two different libraries for all of the structurally characterized tertiary fold templates (29, 30) suggest with high probability scores that, with the exception of a long insertion of 35 amino acids, the Hal3 256–480 polypeptide would display the three-dimensional structure of the homologous proteins AtHal3 and EpD12, 31) (Protein Data Bank codes and sequence identity 1e20 (46%) and 1g63 (26%), respectively). The structure of this group of proteins consists of three protomers, each one folded as a αβ3 protein. Three FMN groups are located in the interface between those protomers. Fig. 7 shows the alignment of the yeast Hal3 256–480 fragment and AtHal3 and its predicted structure as it is output from the modeling servers. In addition, current analytical ultracentrifugation data using recombinant structure as it is output from the modeling servers. In addition, located around 20

**Acknowledgments**—We thank Ramón Serrano for providing Hal3 antibodies. The excellent technical assistance of Anna Vilalta and María Jesús Álvarez is acknowledged.

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