

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

Iván Muñoz^{‡§}, Amparo Ruiz^{‡¶}, Maribel Marquina[‡], Anna Barceló[‡], Armando Albert^{||},
and Joaquín Ariño^{‡**}

From the [‡]Department de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Cerdanyola 08193, Barcelona, Spain and ^{||}Grupo de Cristalografía Macromolecular y Biología Estructural, Instituto de Química Física Rocasolano, Consejo Superior de Investigaciones Científicas, Madrid 28006, Spain

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 (²⁶³KLHVLV²⁶⁸), mutagenesis analysis suggests that this motif is not required for Ppz1 binding and inhibition. The mutation of the conserved His³⁷⁸ (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 His³⁷⁸ 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

* This work was supported by Grants BMC2002-04011-C05-04, GEN2001-4707-C08-03 (to J. A.), and BMC2002-04011-C05-03 (to A. A.) from the Ministerio de Ciencia y Tecnología, Spain and Fondo Europeo de Desarrollo Regional. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains Supplemental Table.

[§] Recipient of a fellowship from the Ministerio de Educación y Cultura.

[¶] Recipient of a fellowship from the Generalitat de Catalunya.

** To whom correspondence should be addressed: Dept. de Bioquímica i Biologia Molecular Facultat de Veterinària, Universitat Autònoma de Barcelona, Cerdanyola 08193, Barcelona, Spain. Tel.: 34-93-5812182; Fax: 34-93-5812006.

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), defined regulatory properties, or/and restricted subcellular location. Such a strategy is particularly important in the case of type 1 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 functions 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 His⁹⁰, a residue conserved in yeast Hal3 (His³⁷⁸). 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.

TABLE I
Yeast strains used in this work

Strain	Relevant genotype	Source/reference
JA100	<i>MATa ura3-52 leu2-3,112 his4 trp1-1 can-1r</i>	9
JC002	JA100 <i>sit4::TRP1 tetO:HAL3</i>	10
JC010	JA100 <i>slt2::LEU2</i>	35
MAR20	JA100 <i>slt2::TRP1 hal3::LEU2</i>	16
JA104	JA100 <i>hal3::LEU2</i>	9
EDN75	JA100 <i>ppz1::KAN</i>	32
IM021	JA100 <i>ppz1::KAN hal3::LEU2</i>	This work
MAR6	1788 <i>MATaα HAL3/hal3::LEU2 VHS3/vhs3::URA3</i>	16
MAR23	JA100 <i>tetO:HAL3 vhs3::URA3</i>	16

could catalyze the decarboxylation of 4'-phosphopantothenoyl-cysteine (13), suggesting that this protein could be involved in coenzyme A biosynthesis, and pointed out an important role of Cys¹⁷⁵ 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 interactions 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 diverse regulatory subunits were of little help to face the question of how *Hal3* might bind to and inhibit *Ppz1*.

To gain insight into the *Hal3* regulatory mechanisms, we have developed a mutagenesis analysis followed by a loss-of-function genetic screen aiming to identify residues relevant for *Hal3* function. The results presented here show that His³⁷⁸ has no function in regulating *Ppz1* activity and allow us to identify a number of residues, most of them clustering between residues 446 and 480, which are important for *Ppz1* binding and/or inhibition. In addition, we provide evidence pointing to possible *Ppz1*-independent functions for *Hal3*.

MATERIALS AND METHODS

Growth Conditions of *Escherichia coli* and *S. cerevisiae* Strains—Except when otherwise indicated, *E. coli* DH5α 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 I.

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).

The source for the *HAL3* gene was plasmid YEp351-*HAL3*, which contains a 2.4-kbp *Bcl*I-*Hind*III DNA genomic fragment (8). Plasmid pGEM3Z-*HAL3* was constructed by digestion of YEp351-*HAL3* with *Eco*RI/*Hind*III and cloning of the 2.4-kbp fragment into the same sites of plasmid pGEM3Z (Promega). Plasmid YEp195-*Hal3* was obtained by cloning the *Eco*RI/*Hind*III *HAL3* fragment into the same sites of YEp195 (23).

Mutation of His²⁶⁵ to glycine was made by PCR using oligonucleotides 5'-*Hal3*H265AR and 3'-*Hal3*HpaI (see Supplemental Table) to amplify a fragment of ~700 bp that was then digested and cloned into the *Bam*HI/*Hpa*I sites of the gapped YEp195-*Hal3* to yield YEp195-*Hal3*(H265G). The mutation of Phe²⁶⁸ to alanine was made in a similar way but using oligonucleotides 5'-*Hal3*F268A and 3'-*Hal3*HpaI. The version of *Hal3* in which His³⁷⁸ was replaced by alanine was made by a sequential PCR strategy using the pairs of oligonucleotides 5'-*HAL3*BamHI/3'-*HAL3*His378Ala and 5'-*HAL3*His378Ala/3'-*HAL3*HpaI in the first step and 5'-*Hal3*BamHI and 3'-*Hal3*HpaI in the second step. The amplification fragment was cloned into the *Bam*HI/*Hpa*I sites of the gapped YEp195-*Hal3* to yield YEp195-*Hal3*(H378A).

Random PCR Mutagenesis and Screen for Loss of *Hal3* Activity—Random PCR mutagenesis was performed essentially as described by Fromant *et al.* (24) using MgCl₂ at a final concentration of 4.7 mM to minimize the occurrence of insertions and/or deletions. YEp351-*HAL3* and oligonucleotides 5'-*Hal3*BamHI_2 and 3'-*Hal3*HpaI_2 were used to amplify the 716-bp fragment between the *Bam*HI and the *Hpa*I 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 *Bam*HI and *Hpa*I 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 *Eco*RI/*Hind*III, and electrophoresed. The 2.4-kbp band was recovered and cloned into YEp195 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-uracil) plates and containing 1 M sorbitol). Approximately 40,000 transformants were plated in CM-uracil 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-uracil medium. They were then replicated in CM-uracil plates and in the same plates containing 1 M sorbitol, 1 M NaCl, 0.2 M LiCl, or 3 mM 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 then were subjected to sequence analysis covering the entire *Bam*HI-*Hpa*I 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 YEp195 by digestion with *Eco*RI and *Hind*III, and the resulting 2.4-kbp frag-

ments were cloned into the same sites of plasmids YCp33 (*URA3* marker), YCp22 (*TRP1* marker), and YEplac 112 (*TRP1* marker).

In Vitro Binding Assays—*In vitro* binding assays were performed as follows. GST-Ppz1 $_{\Delta 1-344}$ 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 YEplac195 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 μ l 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 μ l of 2 \times SDS sample buffer and boiled. Samples (10 μ l) 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'-*HAL3*EcoRI and 3'-*HAL3*XhoI 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-1-thio- β -D-galactopyranoside for induction at 37 °C for 3 h. Conditions of expression and purification of bacterial recombinant GST-Ppz1 $_{\Delta 1-344}$ 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- μ l 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 μ g 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 His³⁷⁸ and the 265HVLV^{F268} 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 HVLV 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 His²⁶⁵ to glycine and Phe²⁶⁸ 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 Phe²⁶⁸ to Ala did not affect the ability of the protein to restore wild type tolerance in a *hal3* strain, whereas a change of His²⁶⁵ 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 *slt2/mpk1* 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 (*sit4 tetO:HAL3*), which suffers a G₁/S blockade in the presence of doxycycline due to lack of *HAL3* expression (10).

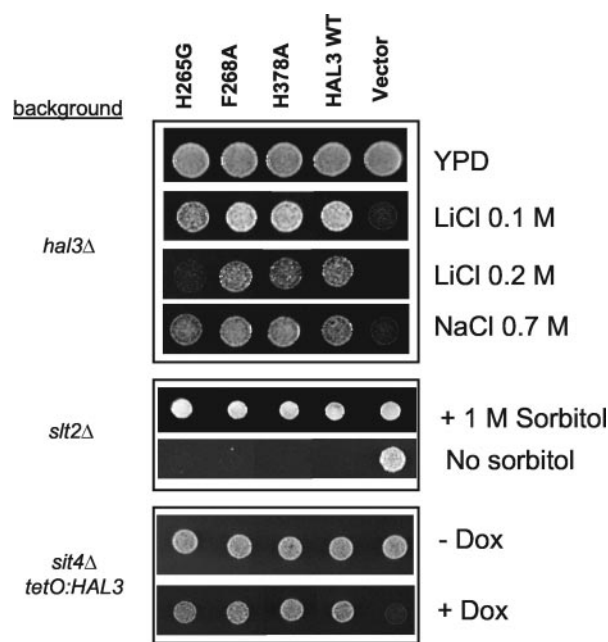


FIG. 1. Effect of mutations on the 265HVLV^{F268} region and of His³⁷⁸ on different functions of Hal3. Upper panel, the centromeric plasmid YCplac33 carrying the indicated versions of Hal3 was introduced in strain JA104 (*hal3*), and cultures were spotted on YPD plates containing the indicated concentration of LiCl or NaCl. Growth was monitored after 36 h. Middle panel, the different versions of Hal3 cloned in the high-copy plasmid YEplac185 were introduced into strain JC010 (*slt2*), and cultures were spotted on synthetic medium (lacking uracil) plates with or without sorbitol and grown for 3 days. Lower panel, plasmids used for the experiment shown in the upper panel were introduced into strain JC002 (*sit4 tetO:HAL3*), and cultures were spotted on synthetic medium (lacking uracil) plates in the presence or absence of 20 μ g/ml doxycycline. Growth was monitored after 2 days.

On the basis of the structure of the AtHal3a protein, it was reasonable to assume that His³⁷⁸ in *S. cerevisiae* Hal3 (equivalent to AtHal3a His⁹⁰) 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 265HVLV^{F268} motif in Hal3 is probably not critical for Ppz1 binding and inhibition. Similarly His³⁷⁸, although theoretically equivalent to AtHal3a His⁹⁰, 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

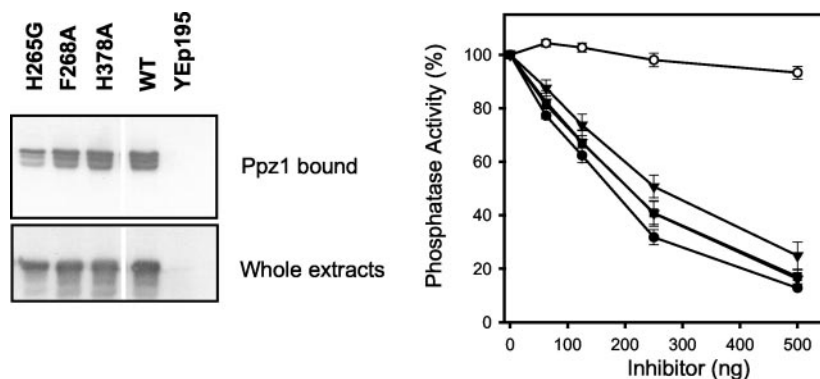


FIG. 2. Analysis of Hal3 versions mutated in the ²⁶⁵HVLV²⁶⁸ region or in His³⁷⁸ for binding and inhibition of Ppz1. Left panel, the bacterially expressed catalytic domain of Ppz1 (Ppz1 Δ_{1-344}) fused to GST was bound to glutathione-agarose beads. Protein extracts from strain IM021 (*ppz1 hal3*) transformed with multicopy plasmids carrying different versions of Hal3 were prepared and incubated with the affinity beads. After extensive washing, the presence of Hal3 in the material retained by the beads was analyzed by immunoblot using anti-Hal3 polyclonal antibodies. The amount of Hal3 in each extract was also evaluated by the immunoblot (lower panel) of 50 μ g of total protein. Right panel, different amounts of bacterially expressed GST (open circles) or GST-Hal3 (closed circles) or the different Hal3 mutated version (H265G, closed triangles; F268A, open triangles; H378A, squares) were incubated with recombinant Ppz1(Δ_{1-344}), and the phosphatase activity was measured as indicated under "Materials and Methods." Values correspond to the mean \pm S.E. from at least three different assays and are expressed as percentage of phosphatase activity relative to control without inhibitors. WT, wild type.

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 Arg²⁵⁶ to Ile⁴⁸⁰ 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 \sim 40,000 colonies yielded around 225 positive clones. The subsequent analysis of the plasmid insert allowed the discarding of \sim 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 carboxyl-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 Ile⁴⁸⁰ 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 Ile⁴⁸⁰ was somewhat less pronounced than that of the wild type protein. The mutation in Val³⁹⁰, 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 Ile⁴⁸⁰ 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

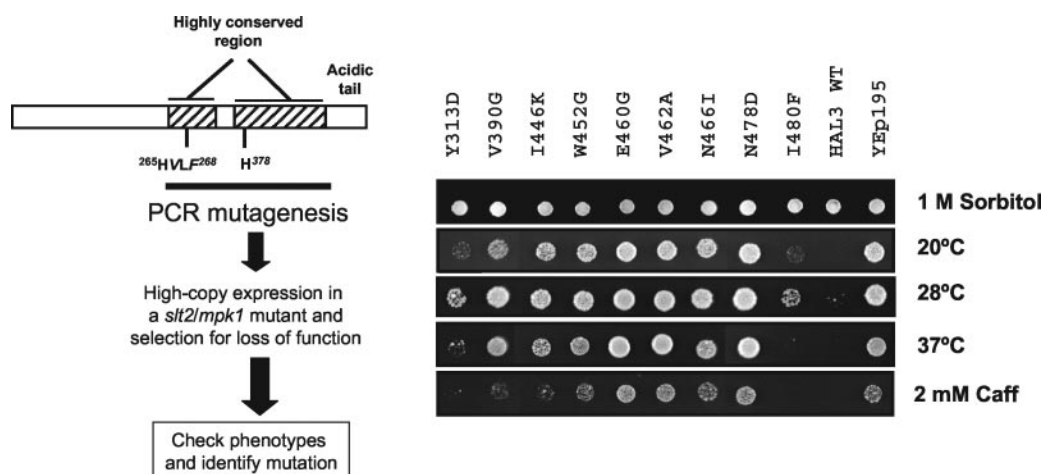


FIG. 3. **Screen for mutations affecting *Hal3* function.** *Left panel*, a schematic depiction of the strategy used to identify residues relevant for *Hal3* function (see “Materials and Methods” for details). *Right panel*, high-copy number plasmids carrying the indicated versions of *Hal3* were introduced into strain JC010 (*slt2*). Cultures spotted in synthetic medium (lacking uracil) plates in the conditions described were grown for 3 days.

TABLE II

Mutations in the Hal3 protein identified by the loss-of-function screen

Numbers in parenthesis denote the number of clones identified for a given mutation.

Amino acid change	Codon mutation
Single changes	
Y313D	TAT to GAT (1)
V390G	GTA to GGA (1)
I446K	ATA to AAA (3)
W452G	TGG to GGG (1)
E460G	GAG to GGG (1)
V462A	GTT to GCT (2)
N466I	AAT to ATT (1)
N478D	AAT to GAT (1)
I480F	ATT to TTT (1)
Multiple changes	
D362G	GAC to GGG
E460G	GAG to GGG (2)
S428P	TCC to CCC
W452G	TGG to GGG (2)
S273P	TCG to CCG
I414V	ATT to GTT
S459P	TCT to CCT (1)

containing 5-fluororotic acid showed that the *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 *Hal3* to complement the chromosomal mutation in a *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 *hal3* and *vhs3* mutations, was transformed with the mentioned versions of *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 *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

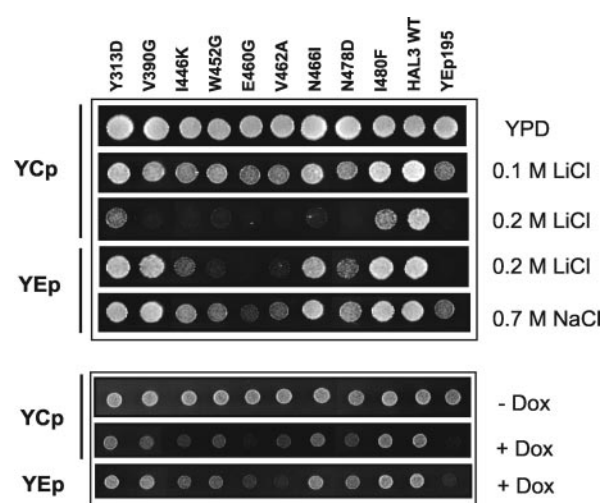


FIG. 4. **Phenotypic effects of the expression of different mutated versions of *Hal3*.** The indicated versions of *Hal3* were introduced as low-copy, centromeric (*YCp*), or high-copy (*YEp*) plasmids in strain JA104 (*hal3*), and the tolerance of the cells to LiCl or NaCl was evaluated after 60 h as in Fig. 1. The same plasmids were used to transform strain JC002 (*sit4 tetO:HAL3*), and growth was monitored after 2 days in the presence or absence of doxycycline (20 μ g/ml). WT, wild type.

markers associated to the mutations plus the plasmid-born marker gene. These experiments confirm our observation in that the *HAL3* and *VHS3* mutations present synthetic lethality (16) and suggest that the mutation of His³⁷⁸ affects some function of *Hal3*, which is required for viability in a *vhs3* background.

The diploid strain carrying any of the five selected mutations within the *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 *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 *Hal3* version to allow survival of a *hal3 vhs3* strain. However, when these cells were tested for their tolerance to saline stress, the mutated *HAL3* versions still displayed the loss-of-function phenotype described in a *hal3* background (compare with Fig. 4). These experiments indicate that *Hal3* mutations able to abol-

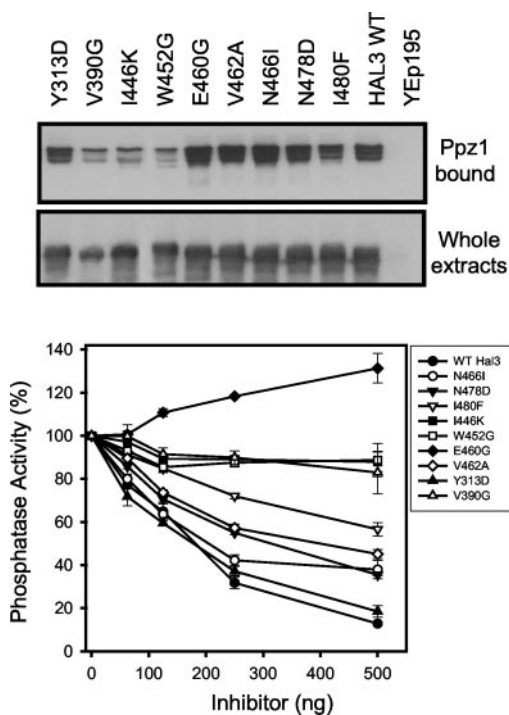


FIG. 5. Ppz1 binding and *in vitro* inhibitory capacity of mutated Hal3 versions identified in the screen. The indicated versions of Hal3 were tested for binding to (upper panel) or inhibitory capacity toward (lower panel) recombinant Ppz1 Δ_{1-344} as indicated in the legend of Fig. 2.

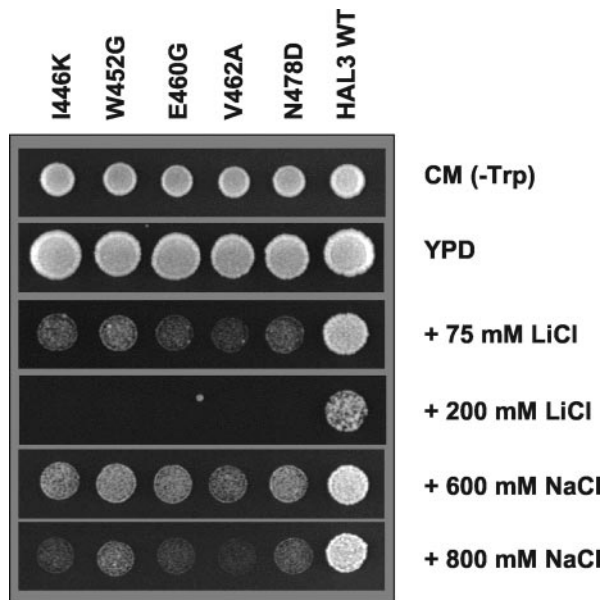


FIG. 6. Complementation of the lethal phenotype of *hal3 vhs3* deletans by Hal3 versions carrying mutations within the 446-478 region. The diploid strain MAR6 was transformed with the indicated versions of Hal3 as a low-copy centromeric plasmid and induced to sporulate. Random spore analysis was performed, and colonies showing markers for both deletions plus the plasmid-born marker gene were selected, recovered, and tested for haploid. Cultures were spotted on the indicated plates and grown for 60 h. WT, wild type.

ish Ppz1 binding or inhibitory capacity do 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 exist-

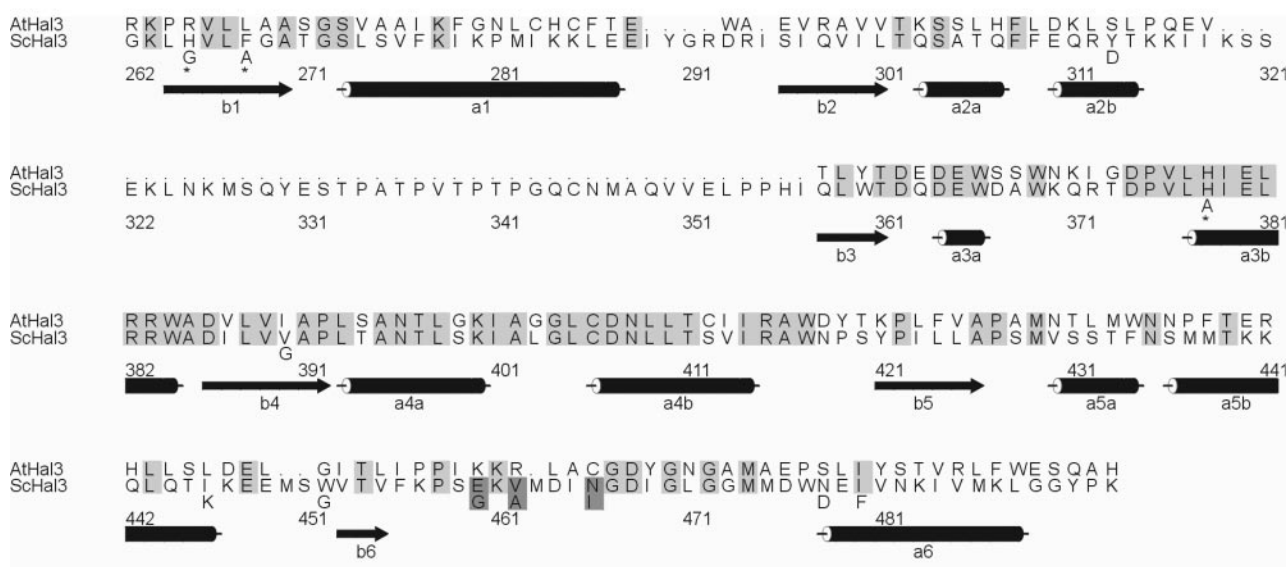
ing evidence that Hal3 binds to the catalytic moiety of Ppz1 even more efficiently than to the entire protein (9), suggesting that the amino-terminal half of Ppz1 might play a protective function against inhibition by Hal3, the molecular basis on how this regulatory subunit can bind and inhibit the phosphatase was unknown. Computer analysis of the Hal3 sequence revealed a $^{263}\text{KLHVLV}^{268}$ motif, which resembles the consensus sequence (R/K)X(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 $^{263}\text{KLHVLV}^{268}$ motif could be relevant for Ppz1 interaction. However, we show that mutation of Phe 268 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 265 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 $^{263}\text{KLHVLV}^{268}$ 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 90 as a residue potentially important in the reaction. The fact that this His is conserved in *S. cerevisiae* Hal3 (His 378) 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 378 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 Arg 256 to Ile 480 , 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 313 to Ile 480 that were relevant for function. Interestingly, the more drastic effects corresponded to changes between Ile 446 and Asn 478 , *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 360 , Ile 446 , and Trp 452 . As expected, these mutations also abolished the ability of Hal3 to inhibit Ppz1 *in vitro*. In contrast, mutations affecting Glu 460 and

A)



B)

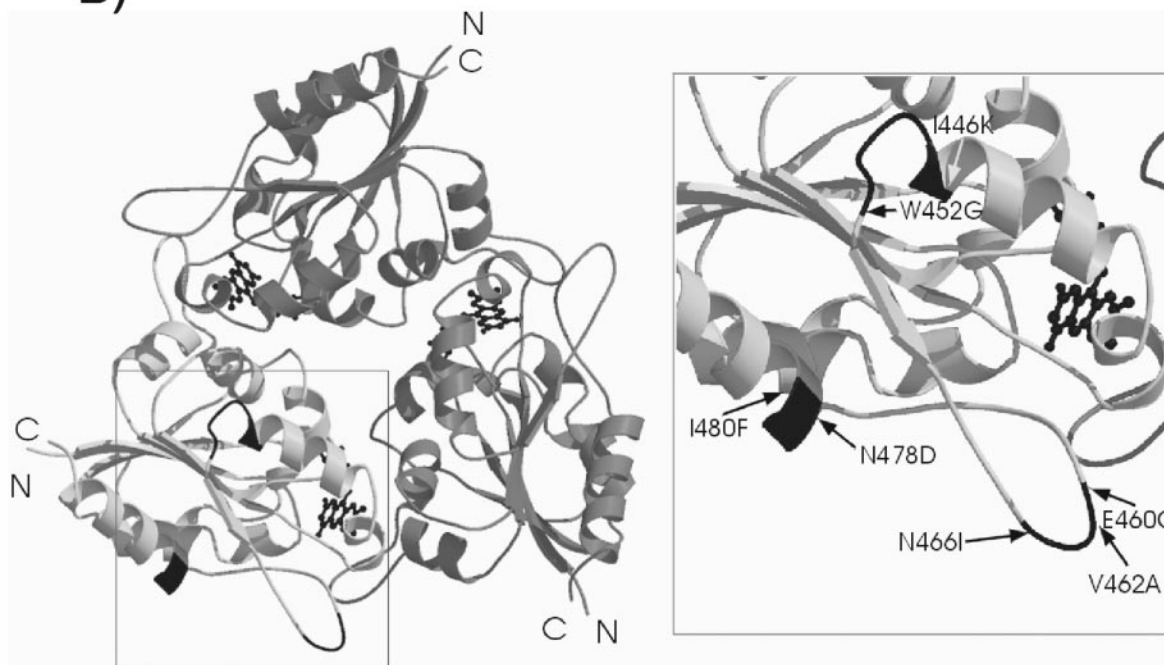


FIG. 7. A, alignment comparing the sequence of AtHal3 and the core domain of yeast Hal3. Residues that are identical are highlighted with a light gray mask. The point mutations are indicated below the Hal3 sequence. Directed mutations are indicated with an asterisk. The predicted secondary structural 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.

Val⁴⁶² did not affect Ppz1 binding but resulted in Hal3 proteins fully (Glu⁴⁶⁰) or partially (Val⁴⁶²) 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

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 can be independent. This finding allows the understanding of our recently reported observation that the Glc7 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 EpiD (12, 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 α/β 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 protein encompassing residues 251–491 (i.e. essentially the same fragment subjected to mutagenesis in this work) support the trimeric structure of the polypeptide (data not shown). As it can be seen in this figure, it is reasonable to use this alignment to predict the position of the point mutations analyzed in this study. As expected, most of the mutations are located in the vicinity of solvent-accessible loop regions; thus, it is likely that they induce a local effect in the surface structure of the protein. On the other hand, V390G is completely buried in the highly conserved hydrophobic core of the protein. Consequently, the observed biochemical properties of this mutant could be caused by an unpredictable effect of several structural changes. Seven of nine single mutants are found in the same area of the macromolecule. This finding suggests a directionality of the interaction between Ppz1 and Hal3. Those seven residues are clustered in three groups. I446K and W452G are located in the loop connecting $\alpha 5$ and $\beta 6$, N478D and I480F are in the NH_2 -terminal moiety of helix $\alpha 6$, and E460G, V462A, and N466I are located around 20 Å apart from the other two groups in an area known as the flap (12, 31). This area has been shown to be unstructured in AtHal3 and EpiD, unless a substrate is bound to the protein (29, 30). Interestingly, the mutations located in the flap do not hinder the interaction with Ppz1, although they inhibit its phosphatase activity. On the other hand, the rest of mutations abolish *in vitro* binding to Ppz1. These findings suggest that yeast Hal3 displays at least two points of interactions with Ppz1 and that regulation of the phosphatase by Hal3 is achieved when both sites are occupied. The Y313D mutation is located close to the above mentioned long insertion of Hal3; thus, it is difficult to predict the effect of the mutation in terms of the modeled structure.

A recent observation in our laboratory is that simultaneous mutation of HAL3 and VHS3 results in a lethal phenotype (16). We report here that His³⁷⁸ is necessary for complementation of the lethal phenotype. Interestingly, we have observed that

mutation of the equivalent His residue in Vhs3 (His⁴⁵⁹) also abolishes the ability of Vhs3 to allow survival of a *hal3 vhs3* strain (16). In contrast, Hal3 His³⁷⁸ (as well as Vhs3 His⁴⁵⁹) is dispensable for other Ppz1-related functions, whereas Hal3 residues necessary for Ppz1 inhibition can be mutated and these versions still allow viable *hal3 vhs3* cells. All of these results suggest that Hal3 (and probably Vhs3) may have Ppz1-independent functions. An important issue would be to understand why the mutation of His³⁷⁸ in Hal3 does not allow the protein to provide Hal3 function in a *hal3 vhs3* mutant. Although at this point we do not know at the molecular level the nature of this genetic interaction as mentioned above, His³⁷⁸ is the residue equivalent to His⁹⁰ in AtHal3a (12). In the plant protein, His⁹⁰ has been proposed to play a key role in a α,β -dehydrogenation reaction involving peptidylcysteine. Further work provided evidence that AtHal3a catalyzes *in vitro* the decarboxylation of 4'-phosphopantotenoylcysteine to 4'-phosphopantetheine, raising the possibility that this enzyme could be involved in coenzyme A biosynthesis (13). Therefore, an exciting possibility would be that the *S. cerevisiae* Hal3 (and possibly Vhs3) could act in the CoA biosynthetic pathway. However, molecular characterization of the AtHal3a catalytic mechanisms (14, 33) has revealed a key role for Cys¹⁷⁵, a residue that is not conserved in Hal3 or Vhs3. Therefore, a hypothetical role of Hal3 or Vhs3 in CoA biosynthesis in *S. cerevisiae*, which would explain the requirement for His³⁷⁸ for function, seems unlikely.

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

REFERENCES

- Cohen, P. T. (2002) *J. Cell Sci.* **115**, 241–256
- Bollen, M. (2001) *Trends Biochem. Sci.* **26**, 426–431
- Posas, F., Casamayor, A., Morral, N., and Arino, J. (1992) *J. Biol. Chem.* **267**, 11734–11740
- Arino, J. (2002) *Eur. J. Biochem.* **269**, 1072–1077
- Yenush, L., Mulet, J. M., Arino, J., and Serrano, R. (2002) *EMBO J.* **21**, 920–929
- Ruiz, A., Yenush, L., and Arino, J. (2003) *Eukaryot. Cell* **2**, 937–948
- Di Como, C. J., Bose, R., and Arndt, K. T. (1995) *Genetics* **139**, 95–107
- Ferrando, A., Kron, S. J., Ríos, G., Fink, G. R., and Serrano, R. (1995) *Mol. Cell. Biol.* **15**, 5470–5481
- de Nadal, E., Clotet, J., Posas, F., Serrano, R., Gomez, N., and Arino, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7357–7362
- Simon, E., Clotet, J., Calero, F., Ramos, J., and Arino, J. (2001) *J. Biol. Chem.* **276**, 29740–29747
- Espinosa-Ruiz, A., Belles, J. M., Serrano, R., and Culiñez-Macia, F. A. (1999) *Plant J.* **20**, 529–539
- Albert, A., Martínez-Ripoll, M., Espinosa-Ruiz, A., Yenush, L., Culiñez-Macia, F. A., and Serrano, R. (2000) *Struct. Fold. Des.* **8**, 961–969
- Kupke, T., Hernandez-Acosta, P., Steinbacher, S., and Culiñez-Macia, F. A. (2001) *J. Biol. Chem.* **276**, 19190–19196
- Steinbacher, S., Hernandez-Acosta, P., Bieseler, B., Blaesse, M., Huber, R., Culiñez-Macia, F. A., and Kupke, T. (2003) *J. Mol. Biol.* **327**, 193–202
- Munoz, I., Simon, E., Casals, N., Clotet, J., and Arino, J. (2003) *Yeast* **20**, 157–169
- Ruiz, A., Munoz, I., Serrano, R., Gonzalez, A., Simon, E., and Arino, J. (2004) *J. Biol. Chem.* **279**, 34421–34430
- Goldberg, J., Huang, H. B., Kwon, Y. G., Greengard, P., Nairn, A. C., and Kuriyan, J. (1995) *Nature* **376**, 745–753
- Egloff, M. P., Cohen, P. T., Reinemer, P., and Barford, D. (1995) *J. Mol. Biol.* **254**, 942–959
- Egloff, M. P., Johnson, D. F., Moorhead, G., Cohen, P. T., Cohen, P., and Barford, D. (1997) *EMBO J.* **16**, 1876–1887
- Zhao, S., and Lee, E. Y. (1997) *J. Biol. Chem.* **272**, 28368–28372
- García-Gimeno, M. A., Munoz, I., Arino, J., and Sanz, P. (2003) *J. Biol. Chem.* **278**, 47744–47752
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Gietz, R. D., and Sugino, A. (1988) *Gene (Amst.)* **74**, 527–534
- Fromant, M., Blanquet, S., and Plateau, P. (1995) *Anal. Biochem.* **224**, 347–353
- Posas, F., Camps, M., and Arino, J. (1995) *J. Biol. Chem.* **270**, 13036–13041
- Treco, D. A., and Winston, F. (1998) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 13.2.10–13.2.11, John Wiley & Sons, Inc., New York
- Wu, X., Hart, H., Cheng, C., Roach, P. J., and Tatchell, K. (2001) *Mol. Genet. Genomics* **265**, 622–635

28. Dombek, K. M., Voronkova, V., Raney, A., and Young, E. T. (1999) *Mol. Cell. Biol.* **19**, 6029–6040
29. Shi, J., Blundell, T. L., and Mizuguchi, K. (2001) *J. Mol. Biol.* **310**, 243–257
30. Kelley, L. A., MacCallum, R. M., and Sternberg, M. J. (2000) *J. Mol. Biol.* **299**, 499–520
31. Blaesle, M., Kupke, T., Huber, R., and Steinbacher, S. (2000) *EMBO J.* **19**, 6299–6310
32. de Nadal, E., Fadden, R. P., Ruiz, A., Haystead, T., and Arino, J. (2001) *J. Biol. Chem.* **276**, 14829–14834
33. Hernandez-Acosta, P., Schmid, D. G., Jung, G., Culianez-Macia, F. A., and Kupke, T. (2002) *J. Biol. Chem.* **277**, 20490–20498
34. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950
35. Vissi, E., Clotet, J., de Nadal, E., Barcelo, A., Bako, E., Gergely, P., Dombradi, V., and Arino, J. (2001) *Yeast* **18**, 115–124