Molecular Insights into the Fungus-Specific Serine/Threonine Protein Phosphatase Z1 in Candida albicans

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ABSTRACT The opportunistic pathogen Candida is one of the most common causes of nosocomial bloodstream infections. Because candidemia is associated with high mortality rates and because the incidences of multidrug-resistant Candida are increasing, efforts to identify novel targets for the development of potent antifungals are warranted. Here, we describe the structure and function of the first member of a family of protein phosphatases that is specific to fungi, protein phosphatase Z1 (PPZ1) from Candida albicans. We show that PPZ1 not only is active but also is as susceptible to inhibition by the cyclic peptide inhibitor microcystin-LR as its most similar human homolog, protein phosphatase 1 (PP1). Unexpectedly, we also discovered that, despite its 66% sequence identity to PP1, the catalytic domain of PPZ1 contains novel structural elements that are not present in PP1. We then used activity and pulldown assays to show that these structural differences block a large subset of PP1/GLC7 regulatory proteins from effectively binding PPZ1, demonstrating that PPZ1 does not compete with GLC7 for its regulatory proteins. Equally important, these unique structural elements provide new pockets suitable for the development of PPZ1-specific inhibitors. Together, these studies not only reveal why PPZ1 does not negatively impact GLC7 activity in vivo but also demonstrate that the family of fungus-specific phosphatases—especially PPZ1 from Candida albicans—are highly suitable targets for the development of novel drugs that specifically target Candida albicans without cross-reacting with human phosphatases.

IMPORTANCE Candida albicans is a medically important human pathogen that is the most common cause of fungal infections in humans. In particular, approximately 46,000 cases of health care-associated candidiasis occur each year in the United States. Because these infections are associated with high mortality rates and because multiple species of Candida are becoming increasingly resistant to antifungals, there are increasing efforts to identify novel targets that are essential for Candida albicans virulence. Here we use structural and biochemical approaches to elucidate how a member of a fungus-specific family of enzymes, serine/threonine phosphatase PPZ1, functions in Candida albicans. We discovered multiple unique features of PPZ1 that explain why it does not cross-react with, and in turn compete for, PP1-specific regulators, a long-standing question in the field. Most importantly, however, these unique features identified PPZ1 as a potential target for the development of novel antifungal therapeutics that will provide new, safe, and potent treatments for candidiasis in humans.

Candida albicans is an opportunistic fungal pathogen that causes candidemia and is the most common cause of health care-associated Candida bloodstream infections in the United States (1). In the past, the majority of candidemia patients were immunocompromised (i.e., individuals with HIV or transplant recipients on immunosuppressant drugs, among others). However, the numbers of nonimmunocompromised patients contracting candididemia have been steadily increasing, with an estimate of 7,000 to 28,000 patients contracting nosocomial candidemia annually (2). Because the mortality rate of candidemia is 40%, these infections result in 2,800 to 11,200 deaths per year. Unfortunately, these numbers are expected to increase as multiple species of Candida are becoming increasingly resistant to antifungal medications, including fluconazole and echinocandins (3). Although a combination of the well-established calcineurin (CN) drugs FK-506 and cyclosporine (CSA) given with the fungal inhibitor fluconazole has resulted in the very potent killing of Candida albicans (4), the immunosuppressant functions of FK-506 and CSA make their use in humans problematic. Furthermore, Candida albicans-specific CN inhibitors are unlikely to be achievable due to the 100% conservation of the CN active and substrate binding sites (5). Given the pressing need for new, potent antifungals, efforts to identify novel protein targets that are essential for virulence and unique to Candida albicans are warranted.

Eukaryotes contain multiple genes that encode serine/threonine protein phosphatase 1 (PP1 [in humans, PP1α, PP1β], and

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PP1 regulates diverse and essential biological processes by dephosphorylating a variety of protein substrates. Although the intrinsic substrate specificity of PP1 is very low, by interacting with regulatory proteins to form distinct holoenzymes (~200 biochemically confirmed PP1 interactors), PP1 achieves high specificity (6–8). More than two decades ago, it was discovered that budding yeast (Saccharomyces cerevisiae), fission yeast (Schizosaccharomyces pombe), and the opportunistic fungus Candida albicans also carry a PP1 gene, coding for GLC7, Dis2, and GLC7-like, respectively (Fig. 1). There is ~80% sequence identity between C. albicans GLC7 and human PP1 isoforms (“GLC7” will be used here to refer to the PP1 homolog in fungal species) (9). Like PP1, GLC7 controls a plethora of essential biological processes, and its activity is regulated by its interaction with multiple regulatory proteins, many of which are conserved in humans (8). However, it was also discovered that fungi express a unique family of PP1-like phosphatases (10, 11). Unlike GLC7, these families of fungus-specific PP1-like phosphatases consist of two distinct domains: an N-terminal domain that is enriched in serines and is predicted to be unstructured (i.e., a member of the intrinsically disordered protein [IDP] family) and a C-terminal catalytic domain that has high sequence similarity to GLC7 (Fig. 1). This suggests that the fungus-specific phosphatases may also bind the GLC7-specific regulatory proteins. This was confirmed in S. cerevisiae, in which yeast two-hybrid studies demonstrated that S. cerevisiae PPZ1 (SdPPZ1) binds some, but not all, S. cerevisiae GLC7 (SGLC7)-specific regulatory proteins (12). However, the molecular determinants that explain why fungus-specific phosphatases bind only a subset of the GLC7-specific regulators are still largely unknown.

Furthermore, not only is PPZ1 important for cation homeostasis and cell wall biosynthesis, it is also critical for C. albicans virulence (13, 14). Namely, deletion of the Ppz1 gene reduces the ability of C. albicans to infect mice, a function that can be rescued by the reintegration of a single copy of the Ppz1 gene. In addition, PPZ1 has also been shown to play a role in the morphological changes in C. albicans associated with infectivity: that is, the transition from the yeast to the hyphal form. This is because the deletion of Ppz1 reduces the rate of hyphal growth (15). Together, these data suggest that the specific inhibition of PPZ1 will prevent this morphological transition and, as a consequence, block C. albicans infectivity without killing the commensal pathogen. The fungistatic effect of such a treatment would be more beneficial than eliminating C. albicans altogether as the latter might result in uncontrolled bacterial proliferation. Here, we used X-ray crystallography and biochemistry to elucidate the structural and functional characteristics of PPZ1 that are unique to the fungus-specific family of phosphatases. We discovered novel structural elements in PPZ1 that not only explain why PPZ1 binds less effectively to GLC7 regulators but also define new interaction surfaces that may be leveraged for the development of novel, effective antifungal therapeutics.

FIG 1 CaPPZ1 is a PP1-like phosphatase. Domain architecture of the PP1-like phosphatases in C. albicans (GLC7-like, PPZ1, and PPQ1/Sal6 [pink]), S. cerevisiae (green), and Homo sapiens PP1α (blue). Fungus-specific PP1-like phosphatases have an N-terminal intrinsically disordered protein domain (IDP [gray]) in addition to a structured C-terminal catalytic domain (pink/green).

RESULTS

PPZ1cat is an active phosphatase with an atypical C terminus.

Like other fungus-specific phosphatases, C. albicans PPZ1 (484 amino acids [aa], 54.4 kDa) has two domains: an N-terminal intrinsically disordered protein (IDP) domain (aa 1 to 170 [PPZ1Nterm]) and a C-terminal catalytic domain (aa 171 to 484 [PPZ1cat]) (14, 16). PPZ1cat and human PP1α exhibit 66% sequence identity throughout their catalytic domains, and the 6 residues that coordinate the active site metals are 100% conserved (Fig. 1; see Fig. S1 in the supplemental material). Accordingly, full-length PPZ1 (PPZ1FL) and its individual domains (PPZ1Nterm and PPZ1cat) are readily isolated from Escherichia coli. In addition, PPZ1Nterm and PPZ1cat are active, as they effectively dephosphorylate a small molecule substrate mimic (p-nitrophenyl phosphate [pNPP]) (see Fig. S2 in the supplemental material). Furthermore, their activities are identical to that of human PP1α purified from E. coli (8) or PP1c (a mixture of PP1 isoforms α, β, and γ) purified from rabbit muscle (17). However, unlike PP1α, which expresses solubly and crystallizes readily without its C-terminal disordered residues (residues 301 to 330) (8), a PPZ1 construct truncated at the corresponding residue (PPZ1catΔ466–484) is largely insoluble compared to PPZ1cat (>10-fold reduction in yield). This suggests that the PPZ1cat C-terminal region is critical for folding and/or stability. Consistent with this conclusion, the secondary structure prediction program PSIPRED (18) predicts that these residues are not disordered as they are in PP1α but instead form an α-helix (see Fig. S3 in the supplemental material).

The C-terminal residues of PPZ1cat are structured and form an α-helix. In order to understand the molecular consequences of the sequence differences between PPZ1 and PP1α and the role of the PPZ1 C-terminal residues in PPZ1 function, we determined the 3-dimensional crystal structure of PPZ1cat to 2.61 Å resolution (Table 1). The PPZ1cat structure includes residues 171 to 478. (The electron density for the last 6 residues, 479 to 484, was not observed, and thus they were not modeled.) As expected, the PPZ1cat structure adopts the canonical PP1 fold (Fig. 2A and B), comprising a mixed α/β protein, whose central loops are positioned to coordinate the active site metals. However, the structures are not identical. The root mean square deviation (RMSD) between PP1α (PDB no. 4MOV [19]) and PPZ1cat is 1.23 Å (backbone). The
TABLE 1  Data collection and refinement statistics

| Parameter | CaPPZ1 | CaPPZ1–microcystin-LR |
|-----------|--------|-----------------------|
| **Data collection** | | |
| Space group | C222₁ | P₃₂₁ |
| Cell dimensions (Å) | 145.0, 183.7, 69.0 | 50.7, 50.7, 201.1 |
| α, β, γ (°) | 90, 90, 90 | 90, 90, 120 |
| Resolution (Å) | 50.0–2.61 (2.66–2.61) | 50.0–2.40 (2.44–2.40) |
| Rmerge (%) | 13.3 (84.5) | 6.6 (19.7) |
| I/σ(I) | 14.1 (2.1) | 30.6 (4.6) |
| Completeness (%) | 99.9 (100.0) | 98.5 (84.2) |
| Redundancy | 5.6 (5.6) | 6.1 (2.7) |
| **Refinement** | | |
| Resolution (Å) | 36.24–2.61 | 43.9–2.40 |
| No. of reflections | 28,311 | 12,283 |
| Rwork/Rfree | 19.4/22.2 | 17.9/23.5 |
| No. of atoms | | |
| Protein | 4,922 | 3,214 |
| Ligand/ion | 32 | 23 |
| Microcystin-LR | NA ¹ | NA ¹ |
| Water | 220 | 50 |
| B-factors | | |
| Protein | 33.0 | 30.8 |
| Ligand/ion | 29.9 | 46.6 |
| Microcystin | NA | 35.5 |
| Water | 30.9 | 30.1 |
| RMSDs | | |
| Bond length (Å) | 0.002 | 0.003 |
| Bond angles (°) | 0.72 | 0.57 |
| Ramachandran plot (%) | | |
| Favored regions | 96.1 | 96.5 |
| Allowed regions | 3.8 | 3.5 |
| Disallowed regions | 0.2 | 0.0 |
| PDB accession no. | 5JPE | 5JPF |

¹ Values in parentheses are for the highest-resolution shell.

The newly widened Z₁-helix binding pocket results in the second significant structural difference between PPZ₁cat and PP₁α: the C-terminal residues of PPZ₁cat are not disordered like they are in PP₁α, but instead form an α-helix that nestles into this newly widened Z₁-helix binding pocket (Fig. 2B to E). This interaction is stabilized by hydrophobic interactions between the C-terminal helix and residues from helices A’ and B, with the interaction centered on PPZ₁ C-terminal helix residue Met473ppz₁ (Fig. 2E). This residue is completely buried from solvent via interactions with Leu464ppz₁, Leu469ppz₁, and Val472ppz₁ from the C-terminal helix as well as by interactions with Phe185ppz₁ from helix A’ and His235ppz₁, Ile238ppz₁, and Arg239ppz₁ from helix B. The majority of these residues are not conserved in PP₁α. In particular, only a single residue (underlined) between the PPZ₁ C-terminal helix and the C-terminal disordered tail of PP₁α is conserved (PPZ₁, 466SAALKVQMKKEKQ478; PP₁α, 306KKNGK-YGQFSGLN313), with the sequence of PPZ₁ consisting of multiple hydrophobic residues and no helix-disrupting glycines (Leu469ppz₁, Val472ppz₁, and Met473ppz₁ versus Gly304pp₁α, Gly307pp₁α, and Gly311pp₁α, respectively), rationalizing why the corresponding residues in PP₁α are unstructured. Finally, the experimental B-factors for residues in the Z₁-helix are higher than the rest of PPZ₁cat (Fig. 2F), suggesting that the Z₁-helix is more dynamic than the rest of the PPZ₁cat.

The Z₁-helix is dynamic. We also determined the structure of PPZ₁cat bound to the marine toxin microcystin-LR (MC [PPZ₁cat–MC]) (Table 1), a potent cyclic peptide inhibitor of PP₁ (20). Both the MC-free and MC-bound structures of PPZ₁cat are highly similar, with an RMSD of 0.54 Å (Fig. 3A). The largest difference is in the B₁2–B₁3 loop, which contains Cys438ppz₁ (Fig. 3B). This cysteine forms a covalent bond with the bound MC identical to that observed in the PP₁α-MC complex (21). This causes the 438CGEFD441 loop to change conformation and become more dynamic (residues 439GEF441 were not modeled in the PP₁α–MC structure due to a lack of density). Unexpectedly, the Z₁-helix is also no longer ordered in the PPZ₁cat–MC structure (Fig. 3A). This was not due to MC binding, but instead the Z₁-helix was displaced by a symmetry-related molecule in the crystal. The observation that the Z₁-helix can be displaced supports the observation that the Z₁-helix is more dynamic than the rest of the PPZ₁cat. Importantly, the positions of L₁ are identical between the PPZ₁cat and PPZ₁cat–MC structures, demonstrating that the conformation of L₁ is intrinsic to PPZ₁cat itself and not a consequence of Z₁-helix binding.
The sequence and structural conservation of regulatory protein binding pockets in PPZ1cat is highly variable. While PP1 exhibits broad specificity, it acts in a highly specific manner by forming stable complexes (holoenzymes) with a host of regulatory proteins that direct PP1 activity toward specific substrates and localize PP1 to specific regions of the cell (8, 22). Recent structural studies have revealed that PP1 binds these regulators using small linear interaction motifs (SLiMs) (7, 19, 23, 24). The most well-

PPZ1cat and 4.4 nM for PP1α (Fig. 3E; see Fig. S2 in the supplemental material).

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FIG 2  The PPZ1-specific C-terminal helix. (A) Sequence alignment of CaPPZ1 (pink) and Homo sapiens PP1 (HsPP1α [blue]) with the observed secondary structural elements indicated above the sequence. Identical residues are indicated by a star, similar residues are indicated by a colon, less similar residues are indicated by a period, and dissimilar residues are indicated by a blank space. Resides from loop 1 (L1) and the PPZ1-specific helix are highlighted in yellow. Arrows (yellow) indicate the hydrophobic residues in the PPZ1-specific helix that are not present in PP1α. (B) The structure of PPZ1 is shown with the secondary structural elements discussed in the text labeled. (C) Overlay of PPZ1 (pink and yellow, as in panel B) and PP1α (blue). The change in conformation of loop L1 between the two structures is indicated by a dashed circle. (D) Stereo image of the overlay between L1 from PPZ1 and PP1α, colored as in panel C. (E) Interactions between the PPZ1-specific C-terminal helix (yellow) and the widened PPZ1-specific helix binding pocket (coral). Residues that make key interactions are shown as sticks and labeled. (F) PPZ1 colored according to residue B-factors, with yellow and green shading indicating higher B-factors.
known is the RVxF SLiM, which is found in ~70% of all known regulators (22). Others include the MyPhoNE, SILK, and Arg SLiMs (6, 7, 19, 23, 25). Mapping of the sequence differences onto the structure of PPZ1cat shows that the bulk of the changes are distally located from the active site (Fig. 4A). This comparison also reveals that the RVxF interaction residues are 91% conserved between PPZ1 and PP1/H9251 (the only difference being a conservative Met290PP1-to-Leu455PPZ1 substitution [Fig. 4B; see Fig. S1 in the supplemental material]), suggesting that all PP1 regulators that contain an RVxF sequence should bind PPZ1. Other sites are similarly conserved. For example, inhibitor-2 (I-2) is a specific protein inhibitor of PP1. In addition to an RVxF and SILK motif, it binds PP1 using a long helix, which binds across the PP1 active site (25). Like the RVxF motif, the I-2 helix binding pocket is highly conserved in PPZ1 (86% identical, 93% conserved [Fig. 4B; see Fig. S1]), also suggesting that this PP1 regulatory protein can bind productively to PPZ1cat.

In contrast, other SLiM binding pockets are much less conserved in PPZ1. For example, the SILK binding sites are only 71% identical and 79% similar between PPZ1 and PP1α (Fig. 4B; see Fig. S1 in the supplemental material). Three of the 4 amino acid differences result in a change from an acidic to uncharged residue (Glu56PP1/Ala221PPZ1, Asp166PP1/Asa331PPZ1, and Glu167PP1/Gly332PPZ1). As these acidic residues coordinate the basic “K” of the SILK motif, the “K” may not be necessary for PPZ1 to bind SILK motif-containing proteins. The same is true for the MyPhoNE binding pocket (23) (Fig. 4B; see Fig. S1). While the majority of residues that define the pocket are largely similar—57% identical and 75% similar—multiple residues differ between the two proteins (Asp179PP1/Val344PPZ1, Gln198PP1/Phe363PPZ1, Gly215PP1/Glu380PPZ1, and His237 PP1/Ser402PPZ1). The Gly215PP1/Glu380PPZ1 substitution results in the largest clash in the superimposed structures, with the Glu380PPZ1 side chain clashing with that of Trp17MYPT1, suggesting that these changes impact MYPT1 binding.

The SLiM binding pocket that is most different between PP1α and PPZ1 is the Arg interaction site, with an identity of 22% and a similarity of 67% (7, 19) (Fig. 4B; see Fig. S1 in the supplemental material). The most significant differences are the replacement of Tyr78PP1α/Val344PPZ1, Gln198PP1α/Phe363PPZ1, Gly215PP1α/Glu380PPZ1, and His237 PP1α/Ser402PPZ1). The Gly215PP1α/Glu380PPZ1 substitution results in the largest clash in the superimposed structures, with the Glu380PPZ1 side chain clashing with that of Trp17MYPT1, suggesting that these changes impact MYPT1 binding.

The SLiM binding pocket that is most different between PP1α and PPZ1 is the ΦΦ interaction site, with an identity of 22% and a similarity of 67% (7, 19) (Fig. 4B; see Fig. S1 in the supplemental material). The most significant differences are the replacement of Tyr78PP1α, which defines the ΦΦ interaction pocket, with Lys243PPZ1, and the replacement of Ala279PP1α with Trp444PPZ1. Both Lys243PPZ1 and Trp444PPZ1 are large, bulky residues that hinder access to the pocket. This suggests that PP1 regulators that contain a ΦΦ motif will bind PPZ1 with lower affinity than PP1α. Finally, although the Arg interaction pocket is perfectly conserved between both PP1α and PPZ1 (see Fig. S1), the presence of the Z1-helix in PPZ1 is expected to negatively impact the binding of...
regulators that use this site for binding, especially those that also use the ΦΦ binding pocket. Because the Z1-helix binds between these two sites, its presence will block access to the Arg site (Fig. 4C), reducing the ability of PPZ1 to bind regulators that contain these sequential SLiMs.

PPZ1 binds only a subset of PP1 regulatory proteins. To test if PPZ1 binds differentially to known PP1 regulators, we used pulldown assays with three well-characterized regulators—GADD34, PNUTS, and spinophilin (Fig. 5A and B). The minimal PP1-binding domains of these regulators bind PP1 with strong affinities (Equilibrium dissociation constant $K_D$ values are 62 nM for GADD34$^{552-567}$ [26], 9.3 nM for PNUTS$^{394-433}$ [19], and 8.7 nM for spinophilin$^{417-602}$ [27]). As predicted, the pulldown assays show that PPZ1$^{cat}$ binds GADD34 less effectively than PP1$^\alpha$ (Fig. 5C and D). Because the RVxF site is nearly perfectly conserved between PP1 and PPZ1$^{cat}$, this suggests that the weakened affinity is largely due to an inability of GADD34$^{552-567}$ to productively bind the ΦΦ motif binding pocket. To quantify the reduction in binding, we used isothermal titration calorimetry (ITC). The affinity of GADD34 for PPZ1$^{cat}$ decreases more than 19-fold, resulting in a $K_D$ of only 1,150 ± 90 nM (Fig. 5E). Consistent with this, peptides that contain only RVxF motifs bind PP1 with $K_D$s in the micromolar range (28), similar to those observed for GADD34$^{552-567}$ and PPZ1$^{cat}$.

While the PP1 binding domain of GADD34 contains only an RVxF motif and ΦΦ motif, other regulators, such as PNUTS$^{394-433}$ and spinophilin$^{417-602}$ contain additional motifs that facilitate PP1 binding (PNUTS, RVxF-ΦΦ-Arg; spinophilin, RVxF-ΦΦ-Arg-spinophilin$^{417-602}$-helix [Fig. 5A and B]). We used pulldown assays to determine if the altered ΦΦ binding pocket and the presence of the Z1-helix negatively impact the binding of these regulators to PPZ1 (Fig. 5C). The results show that PPZ1, compared to PP1$^\alpha$, does not effectively pull down either PNUTS or spinophilin (~85% less binding [Fig. 5C and D]). Furthermore, mutation of either the ΦΦ or Arg motif in spinophilin results in a similar reduction in PP1 binding, to levels similar to those observed for PPZ1 and wild-type spinophilin (see Fig. S4 in the supplemental material). Together, these data demonstrate that the altered ΦΦ-binding pocket coupled with the presence of the Z1-helix (Fig. 5B) negatively impacts the
ability of PPZ1 to interact with regulators that require these sites for binding.

The presence of the N-terminal IDP domain reduces the ability of I-2 to inhibit PPZ1. Unlike GADD34, PNUTS, and spinophilin, I-2 does not bind PP1 at the PPZ1-specific binding pockets. Instead, it binds PP1 using the SILK–RVxF–I-2–helix interaction pockets (25). With the exception of the SILK motif binding pocket, these interaction sites in PPZ1 are largely conserved with those in PP1. Furthermore, the change in the SILK binding pocket suggests that PPZ1 no longer has a strict requirement for the “K” residue (Fig. 4B). This suggests that I-2 will inhibit both PPZ1 and PP1 with equal potencies. To test this, we measured the ability of I-2 to bind and inhibit PPZ1 activity and compared it to that determined for PP1. The data show that both PPZ1 and PP1 are inhibited by I-2 with nearly equivalent IC50s, measured to be 7 and 12 nM, respectively (Fig. 5F). However, unexpectedly, we also discovered that I-2 is a much less potent inhibitor against PPZ1FL, which includes the ~160-amino-acid N-terminal IDP domain (see Fig. S3 in the supplemental material). With PPZ1FL, the IC50 of I-2 increases 39-fold to 278 nM; this increase was observed for both recombinant I-2 and I-2 partially purified from rabbit muscle (not shown). (The latter result is consistent with previous studies [29] that showed that S. cerevisiae PPZ1FL is also poorly inhibited by I-2.) Furthermore, the specific activities of the two PPZ1 constructs also differ significantly, with 4.6 mU/mg for PPZ1FL and 1,200 mU/mg for PPZ1cat. These data

FIG 5 Most PP1 regulatory proteins bind to PPZ1 less effectively. (A) Domain structure of the regulators tested (G34, GADD34552–567; PNUTS, PNUTS394–433; Spino, spinophilin417–602) with their canonical PP1 interaction motifs highlighted in different colors. (B) Structures of the GADD34 (blue; PDB no. 4XPN [26]), PNUTS (orange; PDB no. 4MOY [19]), and spinophilin (gray; PDB no. 3EGG [27]) PP1 holoenzymes, with close-ups of the Z1 helix interaction pocket. (C) The relative binding of PP1α and PPZ1 with the regulators described in panel A was determined using a pulldown assay. (D) Densitometry analysis of the gel in panel B. (E) Binding isotherm of GADD34552–567 with PPZ1cat (Kd, 1,150 ± 90 nM). (F) Dose-response curves of phosphatase activity with increasing concentrations of I-2 with PP1, PPZ1, and PPZ1FL phosphatases. The data represent the means ± SE from 3 experiments.
suggest that the N-terminal domain likely masks one or more binding sites for I-2 (but not the active site, as the PPZ1FL protein is fully susceptible to MC inhibition [Fig. 3E]) and prevents, via an as yet undefined mechanism, PPZ1 inhibition by this protein inhibitor.

**DISCUSSION**

Our structural and biochemical studies reveal why, in spite of their high sequence similarity, fungus-specific phosphatases like PPZ1 bind only a subset of the GLC7-specific regulators (Fig. 6A). Specifically, we discovered that there are three distinct mechanisms by which PPZ1 inhibits the binding of many GLC7-specific regulators. First, sequence differences between PPZ1cat and PP1α in canonical SLIM interaction pockets, especially the Φβ binding pocket, inhibit the subset of regulators that requires these sites for binding (see Fig. S1 in the supplemental material). Second, the presence of the N-terminal IDP domain, which is not found in GLC7, also negatively impacts the binding of at least some regulators (e.g., I-2), likely through an as yet undefined steric mechanism (Fig. 5 and 6A). Third, structural differences between PPZ1cat and PP1α can also negatively impact regulator binding. In particular, our data show that the presence of the Z1-specific helix hinders access to the C-terminal groove by regulators that bind the Arg site via the ΦΦ site (Fig. 5). In addition, the dynamic nature of this helix raises the intriguing possibility that it may also serve a regulatory role in PPZ1 function. Finally, the binding pocket of the Z1-specific helix is also unique, as that of PP5, the only other known serine/threonine protein phosphatase with a C-terminal helix, binds at the front, versus the top, of the core catalytic domain (Fig. 6B) (30). Together, these results show that instead of PPZ1 competing with GLC7 for its regulators, the regulators preferentially bind and control the activity of GLC7 (Fig. 6A). This resolves a long-standing question about why the presence of the fungus-specific phosphatases does not globally disrupt GLC7 function (12).

Our studies also revealed that multiple pockets in PPZ1cat are unique to this phosphatase and thus can be exploited for drug development. While the PPZ1cat and PP1α active sites are perfectly conserved, the conformation of loop L1 is unique in PPZ1cat as is its folded Z1-specific helix. Both of these new structural elements create novel binding surfaces that are not present in human isoforms of PP1. Thus, they are useful for the development of specific, potent antifungals. Because their active sites are perfectly conserved, one strategy for developing a fungus-specific inhibitor of PPZ1 would be to use “fragment linking” (31): i.e., linking a small molecule that targets one of the unique PPZ1-specific binding pockets with a small molecule that, for example, targets the PP1 active site. This would result in an inhibitor that selectively targets PPZ1, as it would bind PPZ1 with higher affinity than human PP1 isoforms. Because PPZ1 is critical for *C. albicans* hypha formation, a morphological change associated with infectivity, this type of PPZ1-specific inhibitor would stop *C. albicans* infections without killing the commensal pathogen. This would prevent uncontrolled bacterial proliferation that can accompany *C. albicans* elimination and thus will result in novel, potent drugs with minimal side effects for the treatment of candidemia.

**MATERIALS AND METHODS**

**Protein expression, purification for pNPP-based assays, and crystallography.** The gene encoding PPZ1cat (aa 171 to 484) was synthesized (GeneArt; Invitrogen), subcloned into the pR1B bacterial expression plasmid (32), and expressed largely as previously described (8). Specifically, the plasmid was cotransformed with the pGRO7 plasmid, which encodes the GroEL/GroES chaperone (TaKaRa), into *E. coli* BL21(DE3) cells (Invitrogen). Cells were grown in LB medium supplemented with 1 mM MnCl₂ at 30°C to an optical density at 600 nm (OD₆₀₀) of ~0.5, at which point arabinose was added (2 g/liter) to induce the expression of the GroEL/GroES chaperone. At an OD₆₀₀ of ~1, the temperature was lowered to 10°C, and the expression of PPZ1 was induced using 0.1 mM IPTG (isopropyl-β-d-thiogalactopyranoside); the protein was allowed to express for ~20 h at 10°C. The cells were harvested by centrifugation, suspended in fresh LB medium (again supplemented with 1 mM MnCl₂ and 200 μg/ml of chloramphenicol to inhibit the ribosome), and agitated for ~5 h at 10°C. Harvested cells were frozen and stored at ~80°C.

All purifications were performed at 4°C. Cells expressing His₄-tagged tobacco etch virus (TEV) protease sequence-PPZ1cat were lysed in lysis buffer (25 mM Tris [pH 8.0], 700 mM NaCl, 5 mM imidazole, 1 mM MnCl₂, 0.1% Triton X-100) using high-pressure homogenization (Avestin G3 EmulsiFlex) in the presence of an EDTA-free protease inhibitor cocktail (Roche). The lysate was clarified by centrifugation at 45,500 × g, filtered through a 0.22-μm pore polyethersulfone (PES) membrane filter (Millipore), and then loaded onto Ni²⁺-nitrilotriacetic acid (NTA) resin (GE Healthcare) pre-equilibrated in buffer A (25 mM Tris [pH 8.0], 700 mM NaCl, 5 mM imidazole, 1 mM MnCl₂). Bound His₄-TEV-PPZ1cat was washed with 100 ml of buffer A, followed by more stringent wash with 100 ml “stringent wash buffer” consisting of 94% buffer A and 6% buffer B (25 mM Tris [pH 8.0], 700 mM NaCl, 250 mM imidazole,
1 mM MnCl\textsubscript{2}). The bound His\textsubscript{6}-TEV-PPZ1\textsubscript{cat} was eluted using 100 mM buffer B and immediately purified by size exclusion chromatography (SEC) using Superdex 75 26/60 preequilibrated in 20 mM Tris (pH 8.0), 500 mM NaCl, 0.5 mM Tris-(2-carboxyethyl)phosphine (TCEP), and 1 mM MnCl\textsubscript{2}. Fractions containing the His\textsubscript{6}-TEV-PPZ1\textsubscript{cat} protein were pooled and incubated overnight with TEV protease at 4°C. A second Ni\textsuperscript{2+}-NTA “subtraction” purification was used to separate cleaved PPZ1\textsubscript{cat} from the cleaved His\textsubscript{6} tag and TEV; the flowthrough, which contained the cleaved PPZ1\textsubscript{cat}, was pooled and purified in a final step using SEC. PPZ1\textsubscript{cat} was pooled, concentrated, and used immediately for crystallization experiments.

**Protein expression and/or purification for dephosphorylation assays.** The catalytic subunit of rabbit skeletal muscle protein phosphatase 1 (PP1) was isolated as described (33). The 20-kDa myosin light chain (MLC20) and the myosin light chain kinase (MLCK) were obtained from turkey gizzard, and MLC20 was phosphorylated by MLCK in the presence of [γ-32P]ATP and Mg\textsuperscript{2+} as described previously (34). Recombinant 6×His-tagged inhibitor-2 (I-2) was prepared as described previously (37) were determined by using either the protein subtrates as a control. The 50% inhibitory concentrations (IC\textsubscript{50}s) of microcystin-LR (obtained from Enzo Life Sciences or purified as previously described (33)) were isolated as described (33). The 20-kDa myosin light chain (MLC20) and the myosin light chain kinase (MLCK) were obtained from turkey gizzard, and MLC20 was phosphorylated by MLCK in the presence of [γ-32P]ATP and Mg\textsuperscript{2+} as described previously (34). Recombinant 6×His-tagged inhibitor-2 (I-2) was prepared as described previously (37); I-2 was also isolated from rabbit skeletal muscle (35) for some control experiments.

The coding sequences for full-length Candida PPZ1\textsubscript{FL} and its conserved C-terminal catalytic domain, termed PPZ1\textsubscript{cat}, were generated by PCR from the pET28-CaPPZ1 plasmid (GenBank accession no. GQ357913 [14, 36]) and cloned into the E. coli expression vector pGEX6p-1 (Amersham Biosciences); both constructs were sequenced (UD Genomed, Ltd.). Both constructs were then transformed into E. coli BL21 (DE3) RIL cells (Agilent), and expression of N-terminal glutathione S-transferase (GST)-tagged proteins was induced with 0.6 mM IPTG (Sigma) at 18°C. Overnight incubation was used to express GST-PPZ1\textsubscript{FL} and its shorter, 3-h incubation period was required to reduce inclusion body formation and ensure optimal production of soluble GST-PPZ1\textsubscript{FL}. In both cases, 0.5 mM MnCl\textsubscript{2} was included in the culture medium, and 1 mM MnCl\textsubscript{2} was present during the subsequent purification steps. The fusion proteins were purified using glutathione-Sepharose 4B (GE Healthcare) resin, and the GST tag was removed using the PreScission protease (GE Healthcare) during elution (following the instructions of the manufacturer). The protein concentration was determined using the Bradford assay, and protein purity was verified by SDS-PAGE.

**Protein phosphatase assays.** The activities of the recombinant C. albicans phosphatases were measured with [32P]-labeled MLC20 substrate as previously described (34), with the exception that the substrate concentration was 1 mM and 2 mM MnCl\textsubscript{2} was included in the assay mixtures. The activity of rabbit PP1 was also determined under identical conditions as a control. The 50% inhibitory concentrations (IC\textsubscript{50}s) of microcystin-LR (obtained from Enzo Life Sciences or purified as previously described [37]) were determined by either the protein substrate or a small molecule substrate as described previously (38), except that pNPP (Sigma-Aldrich) was used instead of 3-O-methylfluorescein phosphatase (OMFP). Reactions were carried out in 96-well plates (Costar). MC concentrations were prepared by serial dilution and added to 150 μl of buffered enzyme (18 mM protein in 40 mM HEPES [pH 7.0], 1.33 mM diithiothreitol [DTT], 1.33% [vol/vol] Triton X-100, 0.133 mg/ml bovine serum albumin [BSA], 1.33 mM sodium ascorbate, 1.33 mM MnCl\textsubscript{2}) in wells containing the reaction mixtures. The high-signal controls were prewashed three times with 500 μl of SEC buffer. Forty microliters of SDS loading buffer was added to the beads, and the samples were boiled at 80°C for 5 min and analyzed on NuPAGE 4 to 12% bis-Tris gels. Gels were stained overnight with SYPRO ruby protein gel stain (Life Technologies) for 5 min and analyzed on NuPAGE 4 to 12% bis-Tris gels. Gels were stained overnight with SYPRO ruby protein gel stain (Life Technologies) according to the manufacturer’s protocols and scanned using a Typhoon 9410 laser scanner (GE Healthcare) with an excitation wavelength of 457 nm and emission filter of 512 nm following destaining. Densitometry was performed using ImageQuant TL 7.0 software for quantification of the band intensity. Accession number(s). Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession no. 5PE for PPZ1 and 5PE for PPZ1-MC.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00872-16/-/DCSupplemental.

Figure S1, TIF file, 2.2 MB.

Figure S2, TIF file, 0.1 MB.

Figure S3, TIF file, 0.1 MB.

Figure S4, TIF file, 1.1 MB.

Figure S5, TIF file, 1.8 MB.

Figure S6, TIF file, 0.1 MB.

Figure S7, TIF file, 0.1 MB.

Figure S8, TIF file, 0.1 MB.

Figure S9, TIF file, 0.1 MB.

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