Histidine Phosphotransfer Proteins in Fungal Two-Component Signal Transduction Pathways

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The histidine phosphotransfer (HPt) protein Ypd1 is an important participant in the Saccharomyces cerevisiae multistep two-component signal transduction pathway and, unlike the expanded histidine kinase gene family, is encoded by a single gene in nearly all model and pathogenic fungi. Ypd1 is essential for viability in both S. cerevisiae and in Cryptococcus neoformans. These and other aspects of Ypd1 biology, combined with the availability of structural and mutational data in S. cerevisiae, suggest that the essential interactions between Ypd1 and response regulator domains would be a good target for antifungal drug development. The goal of this minireview is to summarize the wealth of data on S. cerevisiae Ypd1 and to consider the potential benefits of conducting related studies in pathogenic fungi.

Two-component signal transduction (TCST) pathways regulate many aspects of bacterial life, including stress responses (1, 2), the switch from free-living to biofilm type growth (3–5), cell division (6), and the transition to stationary phase and to sporulation (7). These pathways are most abundant in bacteria with some species sporting over 300 two-component proteins (8). The prototypical bacterial two-component pathway consists of two proteins, a transmembrane sensor histidine kinase (HK) and a soluble response regulator (RR). Most sensor HKs exist in the cell membrane as dimers in which one monomer is able to phosphorylate the other in an initial stimulus-regulated autophosphorylation step (9–13). A phosphotransfer step occurs between the phosphorylated histidine in the sensor histidine kinase and a conserved aspartate within the receiver domain of the RR protein. Phosphorylation of the RR leads to a change in its activity, and an associated or downstream effector domain dictates the nature of the output response. More complex TCST pathways are known in bacteria that include, for example, hybrid proteins with both kinase and receiver domains, more than two proteins in the pathway, and multiple His-Asp phosphotransfer events (14, 15). Phosphotransfer to or from a receiver domain Asp typically involves a histidine-containing phosphotransfer (HPt) domain (15, 16). Although the number of phosphotransfer events in a pathway can vary, the phosphotransfer events in any given pathway culminate in aspartyl phosphorylation and consequent change in response regulator activity.

TCST pathways have been characterized in some detail in fungi, plants, and slime mold. The eukaryotic pathways resemble the more intricate versions of bacterial pathways. Almost all of the eukaryotic two-component pathways involve a hybrid histidine kinase with both kinase and receiver domains (17, 18). In Saccharomyces cerevisiae, the pathway involves autophosphorylation of a membrane-associated histidine kinase followed by an intramolecular phosphotransfer event between the conserved histidine in the HK domain and a conserved aspartate in an attached receiver domain (19). A second step involves transfer of the phosphoryl group on the receiver domain of the hybrid kinase to a conserved histidine on the HPt protein. The final step(s) involves phosphotransfer from the HPt protein to one or more downstream response regulator proteins. Most TCST pathways in eukaryotes have at least two response regulators, one nuclear and one cytoplasmic.

Characterization of various fungal two-component signal transduction pathways has revealed roles for these pathways in osmotic and oxidative stress responses, fungicide sensitivity, phase transition, dimorphism, secondary metabolite production, sporulation, cell wall integrity, hyphal morphogenesis, and sexual and asexual development (20). In addition, two-component pathways are important determinants of pathogenicity in animal pathogens, such as Candida albicans (21, 22), Cryptococcus neoformans (23), Penicillium marneffei (24, 25), and the endemic mycoses, Blastomyces dermatitidis and Histoplasma capsulatum (26), and plant pathogens including Fusarium oxysporum (tomato) (27, 28), Monilinia fructicola (brown rot of stone fruit) (29), Botrytis cinerea (bean, tomato, and apple) (30–32), Alternaria brassicicola (black spot disease on brassicas) (33, 34), Cochliobolus heterostrophus (maize), and Gibberella zeae (cereal) (35). The involvement of two-component pathways in bacterial and fungal pathogenesis has generated significant interest in using these pathways as targets for antimicrobial drug development. Efforts have thus far centered on the histidine kinase protein; however, the HPt and RR domains are also suitable targets, since they are absent from animal genomes.

INNOVATIONS IN TWO-COMPONENT SIGNAL TRANSDUCTION PATHWAYS TO ACCOMMODATE EUKARYOTIC CELLS

Although the basic mechanism of the His-Asp phosphotransfer pathway is conserved, the compartmentalization and larger size of the eukaryotic cell have likely selected for a multistep phosphorelay rather than the simpler two-component pathway that is most common in bacteria. Eukaryotic TCST pathways...
with membrane-associated sensor histidine kinases require the presence of the small free-standing HPt protein, which is capable of shuttling between the cytoplasm and nucleus (36), permitting phosphotransfer to response regulators in different compartments. Of 12,638 nonredundant GenBank sequences (June 2013) containing the HPt domain, 2,177 have the stand-alone HPt domain architecture of the S. cerevisiae protein that has been so thoroughly characterized. Although there are examples of free-standing HPt domains in bacteria (e.g., ShpA and ChpT in Caulobacter crescentus and Spo0B in Bacillus subtilis), most are associated with additional signaling domains (e.g., HK and receiver). Only 14% of bacterial HPt domains (1,729 of 12,022 HPt domain-containing sequences) and 5.5% of archaeal HPt domains (9/162) are free-standing compared to 100% of fungal (177/177), 97% of plant (233/239), and 100% of amoeboida (5/5) (CDART [37]).

Many fungal HKs are not membrane associated and could presumably become nuclearly localized in response to certain stimuli. However, the high conservation in fungal HPt size and structure suggest that this may not be necessary, as the HPt protein can translocate more easily. It will nonetheless be of interest to experimentally determine the localization of the large number of fungal HK proteins predicted to lack transmembrane domains.

Another interesting innovation in the eukaryotic pathways is the interface between TCST proteins and other types of signal transduction pathways. The best-studied example of this is the SLN1 TCST pathway in S. cerevisiae and the HOG1 mitogen-activated protein (MAP) kinase cascade. These pathways are joined by a physical interaction between the Skl1 RR and the mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK) proteins, Sk2 proteins and Sk22 (38, 39). The interaction is regulated by the phosphorylation state of Sk1 (38). The interacting domain in Sk2 was originally defined as the region between amino acids (aa) 294 and 413 (39) and later refined to the 19 amino acid stretch between aa 361 and 380. An SSK2 mutant lacking amino acids 361 to 380 fails to respond to hypersomatic stress (38).

Eukaryotic two-component pathways also feature distinct compartmentalization of the RR proteins. In S. cerevisiae, the Skn7 response regulator is constitutively nuclear, while the Sks1 response regulator is cytoplasmic (36, 40, 41). This organization requires the Ypd1 protein to shuttle in and out of the cytoplasm. In Arabidopsis thaliana, the five AHP-encoded HPt were originally thought to be cytoplasmic with nuclear relocation in response to pathway activation by cytokinin (42, 43). However, recent studies show that the Arabidopsis HPt proteins are localized to both the nucleus and cytoplasm and that this distribution is not responsive to cytokinin (44) consistent with the unregulated nucleocytoplasmic shuttling of S. cerevisiae Ypd1 (36).

EXPANSION OF THE HK AND RR GENE FAMILIES IN EUKARYOTES

In many bacterial genomes, genes of related function like the HK and RR of two-component pathways are organized in operons. This ensures a one-to-one relationship of the components of the pathway. The arrangement of functionally related genes in eukaryotic genomes is less constrained, and this may have contributed to the origin of highly expanded HK and RR gene families in different taxa.

Although the S. cerevisiae genome has a single hybrid HK gene, other fungi have expanded HK gene families with a trend toward higher numbers of HK genes in filamentous fungi. Species of the Saccharomyces class of the Ascomycota have between 1 and 5 HK genes per genome, while the filamentous ascomycetes species have 8 to 16 per genome (18). The plant fungal pathogen Stagonospora nodorum has 19 HK genes (18). Interestingly, the expanded HK gene families in fungi are not predominantly membrane associated. For example, of 254 Ascomycota HK proteins listed in the SMART database (February 2012), only 25 are predicted to have transmembrane (TM) domains, 14 have a single TM domain, and 11 have two, like the S. cerevisiae Sl11 HK. The expansion of the HK gene families may relate to the need for novel sensory activities related to pathogenesis. However, both the nonpathogenic Aspergillus oryzae used in the production of soy sauce, miso, and sake and the pathogenic Aspergillus flavus associated with aspergillosis of the lungs have 14 HK genes (SMART genome database in February 2012).

In contrast, higher plant genomes have a modest HK gene family size with 8 each in Arabidopsis thaliana and Oryzae sativum and 5 in Chlamydomonas reinhardtii, but a highly expanded RR gene family. While most fungal genomes include between 1 and 5 highly conserved RR genes, the Arabidopsis genome has 23 RR-encoding genes and O. sativum has 28, although some of these genes are encoding pseudo-RRs (18, 45), which contain receiver domains but lack key residues required for activity.

Perhaps consistent with the profusion of RR, higher plants also contain more than one Hpt-encoding gene. Arabidopsis contains 5 Hpt genes, and O. sativum has 2. Plant genomes are also known to include divergent Hpt genes, which are presumably incapable of phosphotransfer (18). The imbalance in the size of the HK and Hpt versus the RR gene families suggests that plant RR may mediate non-HK as well as HK-HPt signals.

TAXONOMIC DISTRIBUTION OF HPT GENES

YPD1 orthologs are found in numerous fungal genomes from Ascomycota and Basidiomycota and in the more basal Chytridiomycota (Gonapodya prolifica but not Batrachochytrium dendrobatidis). In addition, a set of paralogous genes were identified in the Rhizopus delmar genome from Zygomycocta (see Fig. 2 and Table 2). No orthologs were found in available Microsporidia or Neo- callimastigomyctes genomes. HK-encoding genes have also been identified in nonfungal genomes, including plants, Dicystostelium, green algae, and diatoms (18, 46). Recent dramatic growth in genomic databases has resulted in the occasional potential misannotation of genes in sporadic animal taxa as belonging to the family of two-component regulators. These misannotations are most common in early assemblies in which short contigs may be maintained until they can be definitively ascribed to contaminating bacterial sequences but could also be attributable to rare horizontal gene transfer events between eukaryotic lineages (47).

BIOLGY OF THE FUNGAL HPT PROTEIN YPD1

S. cerevisiae YPD1 is an essential gene required for transmission of the Snl1 HK signal to the Sk1 RR. Signaling to Sk1 is necessary for viability because Sk1 → P is needed to suppress lethal activation of the HOG1 MAPK pathway under normal osmotic conditions (48). Ypd1 also transmits the Sn1 HK signal to the Skn7 RR; however, viability of the nonphosphoaccepting skn7D427N mutant and the skn7Δ mutants (40, 49) indicate that loss of this activity is not lethal.

Several fungal YPD1 orthologs have been shown to comple-
ment the lethal phenotype of the *S. cerevisiae* *YPD1* mutant, including the *Schizosaccharomyces pombe* *MPR1* gene (also known as *SPY1*), and the *C. albicans* *YPD1* gene, while H/H nonphosphorylatable mutants of *YPD1* orthologs fail to complement (50, 51). This type of *in vivo* data together with the results of *in vitro* phosphorelay reconstitution experiments (52, 53) indicates that *YPD1* orthologs share the TCST phosphotransfer function at the biochemical level. Given the many documented functions of TCST pathways, the *YPD1* orthologs are likely to differ biologically. For example, although the *S. cerevisiae* and *C. neoformans* *YPD1* genes are essential (19, 54), both the *S. pombe* and Dictyostelium genes are dispensable for viability (50, 53, 55, 56). The nonessential *S. pombe* Mpr1 HPt transmits oxidative stress signals to a MAPK pathway (55, 57, 58) and plays a role in regulation of the G2/M cell cycle progression (50) in contrast to the osmotic stress-sensing S. cerevisiae Sln1-Skn7 pathway activation (Fassler, unpublished). Inviable (Fassler, unpublished)

Suppression of the osmotic stress sensitivity of os mutants (59) Iprodione resistance equivalent to os mutant (97)

Wild type and *mprlΔN167* complement *in vivo* and *in vitro* (53)

Precocious entry into M phase (50)

No information

In *vivo* complementation by wild type but not H69Q (51) Reconstitution of phosphorelay from YpdA to RR (52)

**TABLE 1 Phenotypes of fungal hpt mutants**

| Organism | Hpt gene | Deletion phenotype | In vivo phenotype of missense mutant | In vivo phenotype of *ypd1* (*hpt*) | Complementation of *S. cerevisiae* *YPD1* deletion strain *in vivo* or *in vitro* |
|----------|----------|--------------------|-------------------------------------|-------------------------------|-----------------------------------------------------------------|
| *S. cerevisiae* | *YPD1* | In viable (19) | Pradimicin resistance (69) Conditional lethality (Fassler, unpublished) SLN1-SKN7 pathway activation (Fassler, unpublished) | Inviable (Fassler, unpublished) | Not applicable |
| *N. crassa* | *hpt-1* | In viable (59) | | | |
| *S. pombe* | *mpr1*/*spy1* | Viable (50, 55) | | | |
| *C. albicans* | *CaYPD1* | Viable (98) | | | |
| *A. nidulans* | *ypdA* | In viable (60, 99) | | | |

Consistent with the observation of Hog1-independent as well as the essential Hog1-dependent role for Ypd1 signaling known in *S. cerevisiae*, *YPD1* hypomorphs in other fungi exhibit complex phenotypes. For example, Cryptococcus *ypd1Δ hog1Δ* strains, viable because the *hog1* pathway prevents the lethal effects of inappropriate Hog1 pathway activation, exhibit elevated levels of diamide resistance relative to *hog1, ssk1, or skn7* mutants (54). This suggests that Hog1-independent *YPD1*-mediated signaling in Cryptococcus may involve a third RR or some other type of signal. Analysis of viable Neurospora *crassa hpt os-2* double mutants likewise revealed reduced osmotic stress sensitivity and increased oxidative stress sensitivity compared to the *os-2* MAPK mutant, suggesting complex regulation of stress responses that involve both TCST-dependent and TCST-independent regulation (59). Finally, reduction in Aspergillus *nidulans* *ypdA* function, evaluated in *ypdAΔ/ypdA* heterokaryons, caused reduced viability and increased sensitivity to osmotic stress (60), although the dependence of these phenotypes on a downstream MAPK has not been directly tested.

Fungal TCST proteins are important for pathogenesis in both plant and animal fungal pathogens. Many HK genes have been implicated (21, 28, 29, 32, 35, 61); however, the role that His-Asp phosphotransfer plays in pathogenesis has not been clearly established. In the animal pathogen *C. neoformans*, loss of *YPD1* function leads to changes in melanin production, a major virulence factor (54). Additional experiments directly testing the role of Ypd1 or the phosphorylated histidine and phosphoaccepting aspartate in HKs or RR in plant or animal virulence are needed to further establish the requirement for TCST pathway activity in fungal pathogenesis.

**STRUCTURAL FEATURES OF Ypd1 PROTEINS**

*S. cerevisiae* Ypd1 is an all-helical protein with six α-helices and a seventh more compact single-turn 3_10-helix (designated A to G) (Fig. 1) (62). The HPT domain contains a four-helix bundle (αβ-αC-αD-αG) as a minimal core structure. The αC-αD helical hairpin motif of Ypd1 with its centrally located and solvent-exposed histidine is an important conserved structural feature of HPT domains.

Proteins encoded by the fungal *YPD1* gene family range in size from 137 to 295 aa (*Table 2*). The *S. cerevisiae* protein is 167 aa. Ypd1 orthologs in nonfungal eukaryotes are similarly compact. Dictyostelium *RdeA* is 254 aa, and the *Arabidopsis* *Ahp2* protein is 156 aa. Most ascomycetes HPT proteins have short N-terminal extensions of less than 100 aa prior to the start of the HPT domain. *S. pombe* Mpr1 is an exception with an N-terminal extension of 186 aa. Among the basidiomycete HPTs, *Phanerochaete chrysosporium* and *Postia placenta* have relatively short N-terminal extensions, while the *C. neoformans*
and *Puccinia graminis* HPt proteins have longer extensions of 109 and 182 aa, respectively. The role of this N-terminal domain has not been thoroughly investigated, although yeast two-hybrid interaction data suggest that the N-terminal region of the *S. pombe* Mpr1 protein is involved in the interaction with RR receiver domains (53). The sequences of the most conserved portion of the HPt domains corresponding to the fungal Ypd1 orthologs listed in Table 2 were aligned (Fig. 2). Gaps in the sequence alignment fall at the edges or between known secondary structure elements, thus validating the alignment. The *C. neoformans* sequence introduces a small insertion between helices A and B, while *Pichia pastoris* and others introduce small insertions between helices B and C. The spacing between helices C and D is completely conserved, suggesting that the spatial relationship between these two helices or between these helices and other parts of the protein or its interactors may be functionally important.

**Structure-Function Analysis of Ypd1**

Based on structure models of the *S. cerevisiae* Ypd1 protein, a variety of Ypd1 residues in the vicinity of the phosphorylatable H64 are predicted to have important roles in phosphoryl transfer to and from Ypd1. Many of these residues have been functionally characterized (63). For example, an alanine substitution of the highly conserved K67 residue, located one turn of the helix away from H64 in helix C, revealed that K67 is important for efficient histidyl phosphorylation and for His-~P stability (64, 65). K67 is conserved in all aligned fungal Ypd1 orthologs as well as in the HPt from the bacterium *Anaerofustis stercorihominis*, *Arabidopsis* Ahp2, and *Dictyostelium* RdeA (Fig. 2) and is expected to function similarly in all HPt proteins.

In contrast, the positively charged R90 residue in helix D, postulated to be involved in stabilizing the antiparallel arrangement of helices C and D via ionic interactions, was found to have a modest effect on the levels of Ypd1 phosphorylation and a twofold decrease in the stability of the phospho-imidazole linkage but no effect on phosphorelay efficiency (64, 65). Its interaction with Sln1 is normal, and the interactions with Ssk1 and Skn7 are only slightly compromised (66). Interestingly, this position is not conserved among fungi; only 7 of the 32 fungal species in the alignment have K or R at this position (Fig. 2), suggesting that the positive charge at this position may be one of several possible mechanisms for stabilizing helices C and D in fungal Ypd1 proteins.

Analysis of the G68Q substitution mutant confirmed that the small size of glycine at position 68, just 4 residues downstream of H64, is important for access by receiver domains to the H64 residue. The G68Q Ypd1 protein exhibits reduced levels of phosphorylation (63), severe inhibition in receiver domain interactions (66), and no detectable phosphotransfer (64, 65). G68 is conserved in all fungal Ypd1 proteins (Fig. 2). G63, a second conserved glycine adjacent to H64, is also conserved in fungi (Fig. 2). Although the G63 residue has not yet been characterized, the conservation of glycines flanking H64 may suggest that the functionality of a fungal HPt protein requires this negative space/pocket to facilitate its phosphorylation.

The Ypd1-receiver domain interaction surface was also interrogated by alanine-scanning mutagenesis (66, 67) (Table 3). Each mutation was tested for its ability to interact with the Sh1, Ssk1, or Skn7 receiver domain in two-hybrid experiments. A core set of 10 surface residues, including E16, M20, D21, F27, L31, D60, F65, G68, S69, and L63, mapping to helices A, B, and C, were found to be required for all receiver domain interactions. This residue cluster forms a classic hydrophobic binding site for RR docking. Flanking the core set of 10 surface residues are additional residues involved in interactions with specific receiver domains. These residues map to the distal part of helix C and the proximal part of helix D as well as to helices A and B. It remains to be determined which residues dictate the observed Ypd1 phosphotransfer bias for the Ssk1 versus Skn7 RR (68; A. H. West, unpublished data).

Several substitution mutants were tested for their effect on signaling. Point mutations in *YPD1* might cause a partial decrease in phosphotransfer (complete inactivation is lethal). Alternatively, such mutations could cause an increase in phosphotransfer. The G74C mutation was isolated in a directed mutagenesis screen for mutants resistant to the fungicidal
compound pradinicin A (69). Glycine 74 is located in a three-residue reverse turn that connects helix C and helix D and is postulated to be important for structural integrity of the protein. The G74 residue is highly conserved in Ypd1 orthologs (70) that increase signaling. Thus, the G74C mutation enhances Ypd1 phosphotransfer activity rather than diminishing its efficacy due to lack of specificity (75). Recent TCST-directed antibacterial efforts have focused more on the kinase sensory domain and the response regulator domain (76). In fungi, however, simpler high-throughput screens for HK inhibitors have been recently reported. One screen was based on the po-

### TABLE 2 Sequence identifiers and features

| Species                  | gi                  | Other identifier (GenBank, EMBL, or Refseq) | Gene                  | Length (aa) of protein | Hpt domain (aa) |
|--------------------------|---------------------|---------------------------------------------|-----------------------|------------------------|-----------------|
| **Fungal species**       |                     |                                             |                       |                        |                 |
| Ashbya gossypii ATCC 10895 | 44980684            | AAS50589.1                                  | ABL182Cp              | 138                    | 25–90           |
| Aspergillus clavatus NRRL 1 | 121707662           | XP_001271903.1                              | ACLA_049490           | 168                    | 85–109          |
| Aspergillus flavus NRRL3357 | 220699018           | EED55357.1                                  | AFLA_026290           | 166                    | 87–111          |
| Aspergillus fumigatus A1163 | 159125284           | EDF50401.1                                  | AFUB_067390           | 171                    | 61–143          |
| Aspergillus niger CBS 513.88 | 317038304           | XP_004102012.1                              | ANI_1_1060184         | 162                    | 59–109          |
| Aspergillus oryzae IB940 | 317144462           | XP_0001802078.2                             | AOR_1_210154          | 166                    | 87–111          |
| Blastomyces dermatitidis  | 261205810           | XP_002672642.1                              | BDG_02313             | 165                    | 59–145          |
| Coprinopsis cinerea Okayama 7#130 | 299743631          | XP_0002910687.1                             | CCIG_15018            | 229                    | 99–178          |
| Candida albicans          | 9886962             | AF213247.1                                  | CaYPD1                | 184                    | 35–108          |
| Candida dubliniensis CD36 | 223640675           | CAX44979.1                                  | CD36_06780            | 183                    | 35–108          |
| Clavispora lusitaniae     | 170877388           | ACB38709.1                                  | C1YPD1                | 148                    | 38–109          |
| Cryptococcus neoformans   | 58262068            | XP_568444.1                                  | CNM01530              | 209                    | 110–160         |
| Debaryomyces hansenii CBS767 | 50419265          | XP_0451856.1                                | DEHA2C10890p          | 146                    | 35–97           |
| Donopoda prolifera        |                     |                                             |                       |                        |                 |
| Histoplasma capsulatum    | 240281859           | 240281859                                   | HCDG_00941            | 161                    | 33–115          |
| Kluyveromyces lactis NRRL Y-1140 | 50307853     | XP_045920.1                                  | KLLA019338            | 135                    | 32–121          |
| Lachancea thermotolerans  | 255711708           | XP_002552137.1                              | KLTH0B08030p          | 138                    | 34–124          |
| Leptosphaeria maculans    | 312213483           | CBX93565.1                                  | LEMA_P04660.1         | 141                    | 32–95           |
| Lodderomyces elongisporus NRRL YB-4239 | 149247273 | XP_001528049.1                              | LELG_00569            | 243                    | 37–112          |
| Neosartorya fischeri NRRL 181 | 119500460          | XP_001266987.1                              | NFHA_105780           | 171                    | 61–143          |
| Penicillium chrysogenum Wisconsin 54-1255 | 211592212 | CAP98539.1                                  | Pccg12510             | 201                    | 51–133          |
| Penicillium marneffei      | 212542051           | XP_002151180.1                              | PMAA_040507           | 177                    | 54–118          |
| Phanerochaete chrysosporium | 132047         | e_gw02.9411.1                               |                       | 145                    | 12–92           |
| Pichia pastoris CBS 7435  | 328351330           | CCA37729.1                                  | PPT435_Chr2-0030      | 148                    | 42–102          |
| Rhizopus oryzae (delenar)-1 RA 99-880 | 384499124 | EIE8961.5                                  | R03G_14326            | 168                    | 68–147          |
| Rhizopus oryzae (delenar)-2 RA 99-880 | 384485658 | EIE77748                                  | R03G_02452            | 165                    | 40–120          |
| Saccharomyces cerevisiae   | 6319966            | NP_0100461.1                                | ScYPD1                | 167                    | 31–108          |
| Schizosaccharomyces pombe  | 392571352           | CAA22174.1                                  | MPR1                  | 295                    | 187–267         |
| Talaromyces stipitatus ATCC 10500 | 218725057 | EED24474.1                                  | TSTA_07830            | 179                    | 56–136          |
| Trichophyton equinum CBS 127.97 | 326480900      | EGE05001.1                                  | TEQG_03843            | 152                    | 51–133          |
| Vanderwaltozyma polyspora DSM 70294 | 156845926 | XP_0001645852.1                            | Kp01_1054p41          | 137                    | 30–95          |
| Zygosaccharomyces rouxii   | 238940750           | CAR28924.1                                  | ZYROOF15114p          | 163                    | 32–108          |

| Nonfungal species         |                     |                                             |                       |                        |                 |
| Dictyostelium discoideum  | 3513528             | AAC61850.1                                  | RDEA                  | 254                    | 32–112          |
potential for growth inhibition due to activation of the HOG1 osmotic response MAPK pathway by inhibitors of group III HKs from \textit{C. albicans} expressed heterologously in \textit{S. cerevisiae} (77). In this study, several known antifungals were used successfully in proof-of-principle experiments (77). In a related screen, small molecules were tested for fungicidal activity against an \textit{S. cerevisiae} reporter strain expressing a group III HK from \textit{Magnaporthe grisea}. Here, compounds with broad antifungal activity were identified, but these compounds were ultimately shown to be HK independent in their effects (78).

Due to the dearth of safe and effective antifungal drugs, both natural and synthetic peptides have been proposed as new antifungal agents (79, 80). Peptides are a promising class of antifungal agent because they work rapidly with high specificity and can be used in combination with other therapeutic agents. Naturally occurring peptides exhibiting anticryptococcal activity include the membrane active class of antimicrobial peptides (AMP) (81, 82), the human salivary MUC7 mucin peptides (83, 84), and the cationic antimicrobial peptides (85, 86). Synthetic peptides with anticryptococcal activity have also been reported (87, 88). Several examples of protein interaction surfaces that have been specifically targeted with inhibitory peptides are described in recent reviews (89, 90). In each case, the inhibitors were short peptides derived from one of the binding partners. Structural information and data from the \textit{in vivo} and \textit{in vitro} characterization of mutant proteins could be used to rationally design biologically relevant peptides that would inhibit phosphorelay pathways in fungal pathogens like \textit{C. neoformans}. The interactions between the Ypd1 HPT and receiver domain-containing proteins in the SLN1 pathway are essential for viability, and inhibitors that disturb these interactions are predicted to have potential as antifungal drug leads. \textit{Cryptococcus} Ypd1 is an excellent target for antifungal drug design because Ypd1 is a central molecule in fungal TCST pathways and because reduction in Ypd1 activity is expected to compromise fungal fitness, virulence, and viability.

**CONCLUSIONS**

The structurally and genetically well-characterized HPT from \textit{S. cerevisiae} exhibits many attributes of a useful antifungal drug target. It is essential in at least some fungal pathogens, it is a unique, nonredundant protein in all fungal TCST pathways, and it plays an important role in fungal pathogenesis. While the HPT is non-enzymatic, it is nonetheless possible to interfere with the protein interactions that are required for its activity. In \textit{S. cerevisiae} and in...
other fungi for which TCST control of the HOG1 MAPK osmotic response pathway is known. Ypd1 interactions with the upstream HK receiver domain from which it receives a phosphoryl group and with the downstream Ssk1 RR to which it donates a phosphoryl group is essential. The existing co-crystal structures of Ypd1-receiver domain complexes (91, 92) could facilitate the design of such inhibitors.

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**TABLE 3 Structure-function characterization of S. cerevisiae Ypd1**

| Helix† | Residue‡ | Fungal conservation (%)§ | Receiver domain (R1, R2, and R3¶) interactions∥ |
|-------|---------|-------------------------|---------------------------------------------|
| αA    | T12     | X                       | + + +                                       |
|       | I13     | I, V, T ✓               | − − −                                       |
|       | E16     | E or Q                  | − − −                                       |
|       | S19     | X                       | + + +                                       |
|       | M20     | M or L ✓                | − − −                                       |
|       | D21     | ✓                       | − − −                                       |
|       | D23     | D or F ✓                | − − −                                       |
|       | D24     | D or E ✓                | − − −                                       |
| αB    | F27     | ✓                       | − − −                                       |
|       | L31     | L, I, M ✓               | − − −                                       |
|       | Q38     | ✓                       | − − −                                       |
|       | Q45     | X                       | + + +                                       |
|       | R48     | X                       | + + +                                       |
|       | E53     | X                       | + + +                                       |
| αC    | N55     | D, N (EK) ✓             | + + +                                       |
|       | T57     | X                       | + + +                                       |
|       | D60     | S/T (75)                | − − −                                       |
|       | N61     | S (56)                  | + + +                                       |
|       | H64     | ✓                       | + + +                                       |
|       | F65     | F or Y ✓                | − − −                                       |
|       | K67     | K or R ✓                | − − −                                       |
|       | G68Q    | ✓                       | − − −                                       |
|       | S69     | ✓                       | − − −                                       |
|       | S70     | S or A ✓                | − − −                                       |
|       | L73     | ✓                       | − − −                                       |
|       | G74C    | ✓                       | Not tested                                   |
| αD    | Q76     | S/T (50)                | − − −                                       |
|       | W80     | X                       | − − −                                       |
|       | E83     | ✓                       | − − −                                       |
|       | Q86     | ✓                       | + + +                                       |
|       | R90     | X                       | + + +                                       |

† Shaded rows indicate residues residing in the designated helix. αA, α-helix A.
‡ Residue numbering and identity are based on the S. cerevisiae protein. The residue was substituted with alanine except where indicated.
§ Conservation was evaluated for 32 fungal species listed in the alignment in Fig. 2. The amino acids conserved in fungi are shown. The percent conservation is shown in parentheses. “X” indicates that the residue is not conserved (<25%), a check mark indicates the residue is completely conserved (100%), and a check mark next to a set of residues indicates there is more than one conserved residue at that position.
¶ R1, R2, and R3 refer to the Sln1, Skl1, and Skn7 receiver domains, respectively.
∥ Interactions were tested by two-hybrid assays ([66, 67].

H.64a was used in the interaction assays, and H.64q was used in the biochemical assays.
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