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MINIREVIEW

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 brassiciola* (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

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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 standalone 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 amoebozoa (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 Ssk1 RR and the mitogen-activated protein kinase kinase kinase (MAPKKK) proteins, Ssk2 and Ssk22 (38, 39). The interaction is regulated by the phosphorylation state of Ssk1 (38). The interacting domain in Ssk2 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 hyperosmotic 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 Ssk1 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 HPts were originally thought to be cytoplasmic with nuclear relocalization 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 *Saccharomycetes* 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* Sln1 HK. The expansion of the HK gene family 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 RRs, 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 RRs 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 prolifera* but not *Batrachochytrium dendrobatidis*). In addition, a set of paralogous genes were identified in the *Rhizopus delemar* genome from Zygomycota (see Fig. 2 and Table 2). No orthologs were found in available Microsporidia or Neocallimastigomycota genomes. HPt-encoding genes have also been identified in nonfungal genomes, including plants, *Dictyostelium*, 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).

BIOLOGY OF THE FUNGAL HPt PROTEIN Ypd1

S. cerevisiae YPD1 is an essential gene required for transmission of the Sln1 HK signal to the Ssk1 RR. Signaling to Ssk1 is necessary for viability because Ssk1~P is needed to suppress lethal activation of the Hog1 MAPK pathway under normal osmotic conditions (48). Ypd1 also transmits the Sln1 HK signal to the Skn7 RR; however, viability of the nonphosphoaccepting *skn7D427N* mutant and the *skn7*Δ mutants (40, 49) indicate that loss of this activity is not lethal.

Several fungal *YPD1* orthologs have been shown to comple-

TABLE 1 Phenotypes of fungal *hpt* mutants

Organism	HPt gene	Deletion phenotype	*In vivo* phenotype of missense mutant	*In vivo* phenotype of *ypd1* (*hpt*) *hog1* pathway double mutant	Complementation of *S. cerevisiae ypd1* deletion strain *in vivo* or *in vitro*
S. cerevisiae	*YPD1*	Inviable (19)	Pradimicin resistance (69) Conditional lethality (Fassler, unpublished) SLN1-SKN7 pathway activation (Fassler, unpublished) Inviable (Fassler, unpublished)		Not applicable
N. crassa	*hpt-1*	Inviable (59)		Suppression of the osmotic stress sensitivity of *os* mutants (59) Iprodione resistance equivalent to *os* mutant (97)	No information
S. pombe	*mpr1/spy1*	Viable (50, 55)		Defective oxidative stress signaling (55) Precocious entry into M phase (50)	Wild type and *mpr1*ΔN167 complement *in vivo* and *in vitro* (53)
C. albicans	*CaYPD1*	Viable (98)			*In vivo* complementation by wild type but not H69Q (51)
A. nidulans	*ypdA*	Inviable (60, 99)		No mutant analysis reported	Reconstitution of phosphorelay from YpdA to RR (52)

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/Q 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 cascade (55, 57, 58) and plays a role in regulation of the G_2/M cell cycle progression (50) in contrast to the osmotic stress-sensing function of *S. cerevisiae* Ypd1. Table 1 summarizes the phenotypes of fungal *hpt* mutants. In general, two-component pathways involved in osmoregulation via the Hog1 MAPK signaling have an essential function.

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 ypd1*Δ *hog1*Δ strains, viable because the *hog1* mutation 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 signaling. 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 *ypdA*Δ/*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 RRs 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 (αB-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*

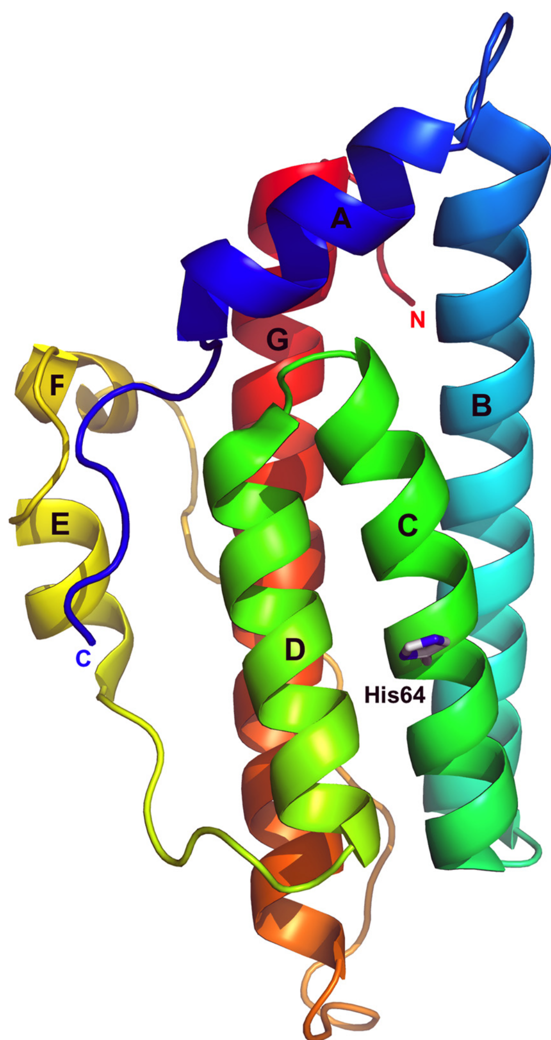

FIG 1 Ribbon representation of Ypd1. The core of the Ypd1 molecule is a compact four-helical bundle composed of the α-helix B (αB), αC, αD, and αG helices. Residues from Ypd1 that make contact with Sln1 come from helices αA, αB, αC, and αD (66, 91). The H64 side chain of Ypd1 that is involved in phosphotransfer is shown as a stick model.

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 Sln1, 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

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	EDP50401.1	AFUB_067390	171	61–143
Aspergillus niger CBS 513.88	317038304	XP_001402021.2	ANI_1_1060184	162	59–109
Aspergillus oryzae RIB40	317144662	XP_001820278.2	AOR_1_2120154	166	87–111
Blastomyces dermatitidis	261205810	XP_002627642.1	BDBG_02313	165	59–145
Coprinopsis cinerea Okayama 7#130	299743631	XP_002910687.1	CC1G_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_458156.1	DEHA2C10890p	146	35–97
Gonapodya prolifera		JGI-128095		175	53–133
Histoplasma capsulatum	240281859	240281859	HCDG_00941	161	33–115
Kluyveromyces lactis NRRL Y-1140	50307853	XP_453920.1	KLLA0D19338g	135	32–121
Lachancea thermotolerans	255711708	XP_002552137.1	KLTH0B08030p	138	33–124
Leptosphaeria maculans	312213483	CBX93565.1	LEMA_P044660.1	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	NFIA_105780	171	61–143
Penicillium chrysogenum Wisconsin 54-1255	211592212	CAP98539.1	Pc22g12510	201	51–133
Penicillium marneffei ATCC 18224	212542051	XP_002151180.1	PMAA_040370	177	54–118
Phanerochaete chrysosporium	132047	e_gww2.9.411.1		145	12–92
Pichia pastoris CBS 7435	328351330	CCA37729.1	PP7435_Chr2-0030	158	42–102
Rhizopus oryzae (*delemar*)-1 RA 99-880	384499124	EIE89615.1	RO3G_14326	168	68–147
Rhizopus oryzae (*delemar*)-2 RA 99-880	384485568	EIE77748	RO3G_02452	165	40–120
Saccharomyces cerevisiae	6319966	NP_010046.1	ScYPD1	167	31–108
Schizosaccharomyces pombe	3925752	CAA22174.1	MPR1	295	187–267
Talaromyces stipitatus ATCC 10500	218725057	EED24474.1	TSTA_078330	179	56–136
Trichophyton equinum CBS 127.97	326480990	EGE05000.1	TEQG_03843	152	51–133
Vanderwaltozyma polyspora DSM 70294	156845926	XP_001645852.1	Kpol_1054p41	137	30–95
Zygosaccharomyces rouxii	238940750	CAR28924.1	ZYRO0F15114p	163	32–108
Nonfungal species					
Dictyostelium discoideum	3513528	AAC61850.1	RDEA	254	32–112

compound pradimicin 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 (Table 3 and Fig. 2). Substitution of G74 to C was postulated to alter Ypd1 structure, causing resistance to pradimicin and sensitivity to osmotic stress (69). *In vivo* SLN1 pathway reporter gene assays using a *SKN7*-dependent *lacZ* reporter showed that this mutation increased signaling. Thus, the G74C mutation enhances Ypd1 phosphotransfer activity rather than diminishing it (J. S. Fassler and Y. Igarashi, unpublished results). The osmosensitivity of this *YPD1* mutant is consistent with previous observations of osmosensitivity in *SLN1* mutants (*sln1**) that increase signaling (70) and is presumably due to reduction of the dephosphorylated form of Ssk1 required for activation of the Ssk2/22 MAPKKKs in the Hog1 MAPK pathway. Pradimicin resistance may likewise be a function of loss of Ssk1 signaling to Hog1 and to Ssk1-dependent cell death pathways.

Several additional *ypd1* mutants were tested for their viability, pradimicin resistance and signaling phenotypes. Of these *ypd1* mutants, the F27A and L73A mutants were viable and exhibited both pradimicin resistance and pathway activation. Other mutants tested (R48A, Q76A, and Q86A) were pradimicin sensitive and showed no pathway activation (J. S. Fassler, unpublished results). Interestingly, mutants exhibiting activation phenotypes were defective in all (three) receiver domain interactions. Hence, the observed changes in activity do not correlate well with two-hybrid experiments (66, 67). It will be of interest to structurally characterize the Ypd1 protein from one or more of the activated mutants.

PROSPECTS FOR Ypd1 AS AN ANTIFUNGAL DRUG TARGET

Previous screens for inhibitors of bacterial TCST pathways have been based on detection of the autophosphorylated HK protein or the phosphorylated RR protein (71–74). Of the inhibitors identified in this way, some failed to inhibit growth of microbial test strains (74), and others were found to be inadequate 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-

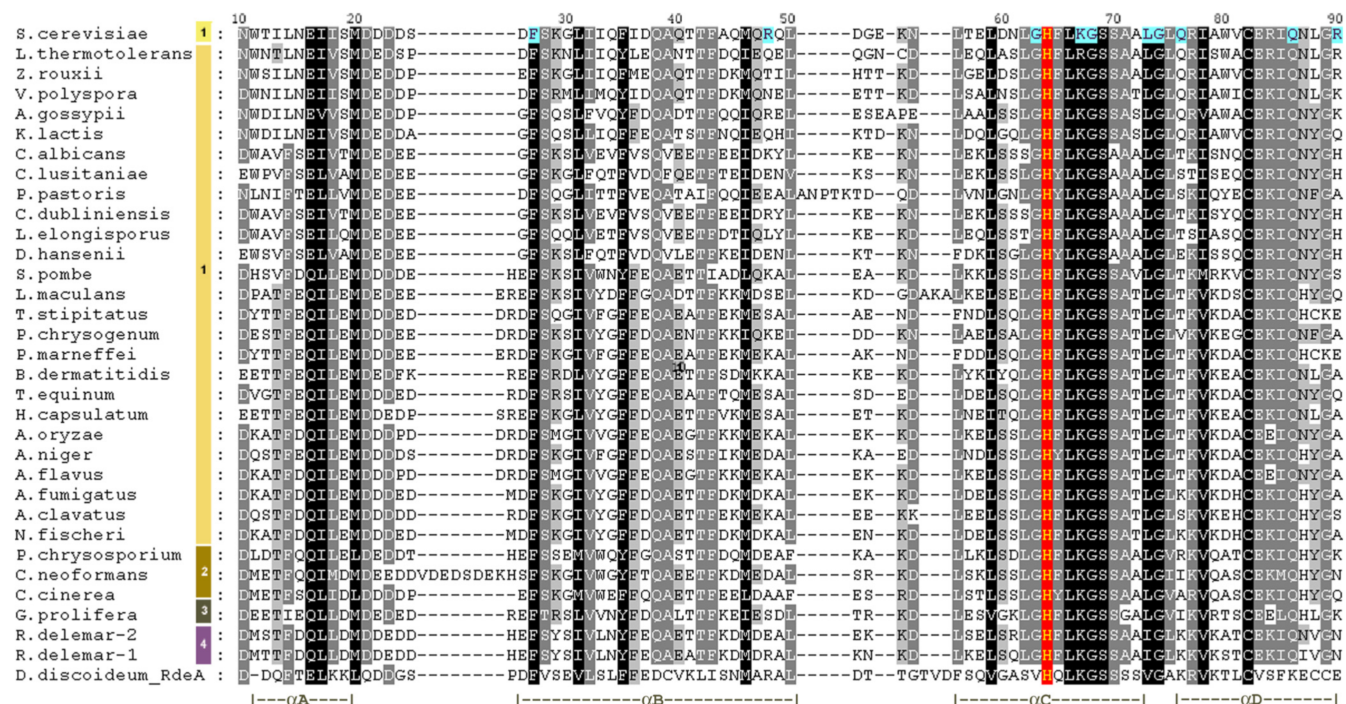

FIG 2 Multiple-sequence alignment of the HPt domain of diverse fungal Ypd1 orthologs. Orthologs were retrieved by BLASTp analysis using the National Center for Biotechnology Information (NCBI) and Joint Genome Institute (JGI) web servers and confirmed when possible using orthology calls from the Ensembl Fungi database and phylogenetic analysis (not shown). The full species names, NCBI gi numbers, other accession numbers, and systematic gene names are provided in Table 2. Complete peptide sequences were aligned using MUSCLE (93). Aligned sequences were imported into GeneDoc (94). Four levels of conservation with default conservation groups enabled were used and are indicated as follows: 100% conservation, black background; 80% conservation, gray background with white lettering; 60% conservation, gray background with black lettering; less than 60% conservation, white background). The image was then imported into Adobe Photoshop for further annotation. Taxonomic representation is indicated using color and numerical codes shown to the left of the sequence alignment: *S. cerevisiae* (light yellow 1); other Ascomycetes (dark yellow 1); Basidiomycetes (mustard 2), Chytrids (brown 3); Zygomycetes (magenta 4). The numbering across the top corresponds to the residues in the *S. cerevisiae* Ypd1 protein. The conserved phosphoaccepting histidine (H64 in *S. cerevisiae*) is shown using a red background. Additional residues in the *S. cerevisiae* protein that have been genetically or biochemically characterized and are discussed in the text are shown on a blue background. Secondary structure elements derived from the *S. cerevisiae* Ypd1 protein structures (Protein Data Bank [PDB] identifications [IDs] 1QSP and 1CO2) (95, 96) are shown across the bottom of the alignment. Gaps introduced to maximize alignment are indicated by dashes.

tential for growth inhibition due to activation of the HOG1 osmotic response MAPK pathway by inhibitors of group III HKs from *C. albicans* expressed heterologously in *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 *S. cerevisiae* reporter strain expressing a group III HK from *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 *in vivo* and *in vitro* characterization of mutant proteins could be used to rationally design biologically relevant peptides that would inhibit phosphorelay pathways in fungal pathogens like *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. *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 *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 nonenzymatic, it is nonetheless possible to interfere with the protein interactions that are required for its activity. In *S. cerevisiae* and in

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

Helix[a]	Residue[b]	Fungal conservation (%)[c]	Receiver domain (R1, R2, and R3[d]) interactions[e]
αA	T12	X	+ + +
	I13	I, V, T ✓	+ − −
	E16	E or Q	− − −
	S19	X	+ + +
	M20	M or L ✓	− − −
	D21	✓	− − −
	D23	D or E ✓	+ + −
	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[f]	✓	+ + +
	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	+ + +

[a] Shaded rows indicate residues residing in the designated helix. αA, α-helix A.
[b] Residue numbering and identity are based on the *S. cerevisiae* protein. The residue was substituted with alanine except where indicated.
[c] 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.
[d] R1, R2, and R3 refer to the Sln1, Ssk1, and Skn7 receiver domains, respectively.
[e] Interactions were tested by two-hybrid assays (66, 67).
[f] H64A was used in the interaction assays, and H64Q was used in the biochemical assays.

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 will be essential. The existing cocrystal structures of Ypd1-receiver domain complexes (91, 92) could facilitate the design of such inhibitors.

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