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MINIREVIEW

Two-Component Signal Transduction Proteins as Potential Drug Targets in Medically Important Fungi[▽]

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Treatment of diseases such as invasive candidiasis and invasive aspergillosis (IA) remains problematic for the clinician. Costs of patient care management are staggering and are most often associated with an increased length of stay in the hospital associated with a delayed therapeutic intervention and other problems (3, 45, 54, 70, 71). But IA and invasive candidiasis are only two of several clinically relevant fungal diseases. A significant number of infections are common among healthy populations, including vulvovaginal candidiasis (61). Also, cryptococcosis occurs in human immunodeficiency virus/AIDS patients, especially in developing countries, but also has been reported in an outbreak that likely included mostly healthy individuals (33). The dimorphic, endemic fungi are also major pathogens of otherwise healthy individuals. For example, the incidence of coccidioidomycosis alone is about 100,000 cases per year (24). Immunocompromised patients are at risk for these diseases also, and in fact, a 5 to 7% crude mortality rate has been observed in hospitalized patients (20). Further, the endemic mycoses like histoplasmosis can present as common-source or focal epidemics, which can result in disease in a significant number of patients (59). The extensive and expanding list of fungal pathogens and the frequency of their occurrence demand the availability of drugs to counter disease.

New antifungal drugs are sought because the former "gold standard," amphotericin B (binds to membrane ergosterol causing changes in permeability), invariably causes toxicity in the patient, negating the importance of its fungicidal activity. Triazoles (target ergosterol synthesis) are now more often used in treatment of fungal disease given their reduced toxicity and in many cases ease of treatment. However, the emergence of new species (*Candida* species other than *Candida albicans*) among clinical isolates is due to their lack of susceptibility to the triazoles. The β-1,3-glucan inhibitors (caspofungin and micafungin) are fungicidal but ineffective against *Cryptococcus neoformans* and of questionable value in IA patients (67). Terbinafine (an allylamine that targets ergosterol synthesis) offers promise although it currently is recommended only for superficial fungal infections.

Drug discovery is currently based upon the paradigm that a target must be a growth-essential gene product. This review is intended to suggest that compounds that inhibit virulence factors of fungal pathogens need consideration for new antifungal drug discovery. This hypothesis was recently discussed in regard to antibacterial drug discovery (15). Species-specific virulence factors of human fungal pathogens such as the capsule of *Cryptococcus neoformans* are known. But we will develop the theme that a conserved signal transduction pathway that regulates the expression of virulence factors across fungal pathogens but that is not found in humans could represent a target for drug discovery. We distinguish "virulence-essential" from "growth-essential" gene products since most in the former category are not required for growth in vitro. Specifically, this review will focus upon two-component proteins that are critical to a number of processes fungi pathogenic to humans use to adapt to the host environment.

First described for both pathogenic and environmental, nonpathogenic bacteria, the term "two component" reflects a requirement for two proteins, one a histidine kinase (HK), usually a transmembrane protein that autophosphorylates using ATP upon perception of an environmental cue (47). Phosphorelay is accomplished on a response regulator (RR) protein, which usually acts as a transcription factor to adapt cells to the environmental signal. A major difference between bacteria and lower eukaryotes is that the latter usually (but not always) require an intermediate protein, a histidine phosphotransfer protein (Hpt), which shuttles phosphate from the HKs to RR proteins. The classic pathway which has been studied extensively in fungi is the HOG1 (hyperosmotic glycerol) mitogen-activated protein kinase (MAPK) pathway (30). Regulation of the HOG1 MAPK pathway requires three upstream proteins that participate in a phosphotransfer relay, including Sln1p (a transmembrane HK), Ypd1p (a cytoplasmic Hpt), and Ssk1p (an RR protein). In addition, other HKs and at least one other RR are found in a variety of fungi, and those fungi pathogenic to humans are depicted in Fig. 1A and B. Most domain functions indicated for each protein are inferred from studies of model fungi.

Curiously, in the absence of stress, phosphotransfer among Sln1p-Ypd1-Ssk1p occurs but activation of the HOG1 MAPK does not since the phosphorylated RR protein Ssk1p is unable to activate the Ssk2p MAPK kinase kinase of the HOG1 MAPK pathway, at least in *C. albicans* and *Saccharomyces cerevisiae* (Fig. 2A). There are sound reasons for this, including the fact that, in the absence of stress, cellular machinery is

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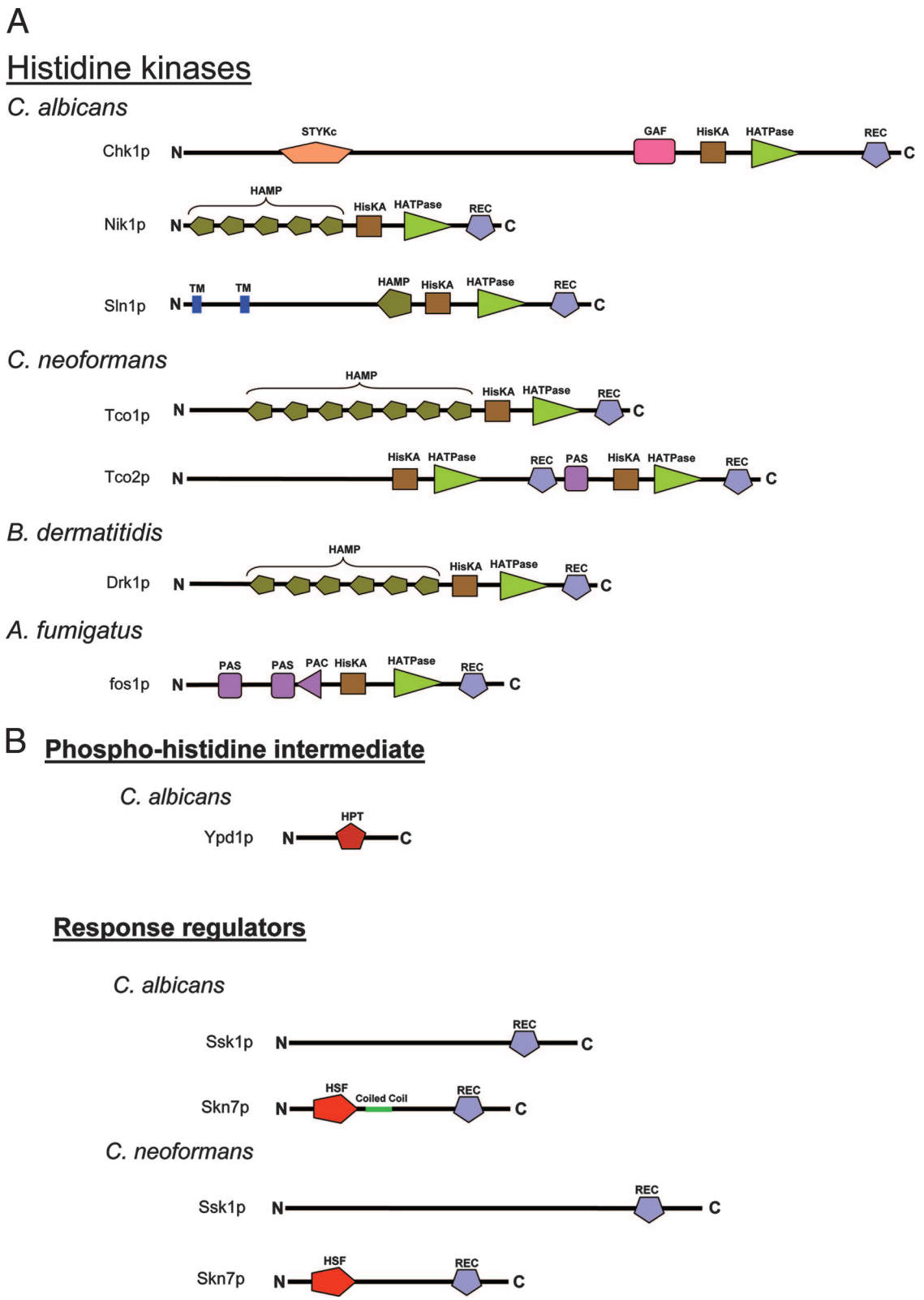

FIG. 1. Two-component signal proteins of selected fungal pathogens. (A) Domains of HKs from fungi pathogenic to humans. Black lines represent the relative sizes of the proteins. GAF, cyclic GMP phosphodiesterase/adenylcyclase/FhlA; HAMP, hepcidin antimicrobial peptide; HATPase, ATPase with dephosphorylation of histidine residues; HisKA box, phosphorylation domain containing the histidine (H) residue; HSF, heat shock factor; PAS, sensor domains of light, redox potential, or oxygen; PAC, C-terminal motif to PAS, which contributes to the structural activity of PAS; Rec, RR (receiver) domain, a putative site of aspartate phosphorylation; STYKc, serine threonine MAPK; TM, transmembrane. (B) Phosphohistidine intermediate and RR proteins in selected fungi pathogenic to humans. HSF, coiled-coil domains, and receiver (rec) domains are shown for each protein. Hpt, site of histidine phosphotransfer between an HK and an RR protein.

TABLE 1. Selected HKs of medically important fungal pathogens

Organism(s)	HK	Protein features[a]	Functions	Reference(s)
B. dermatitidis and *H. capsulatum*	Drk1p	1,274 aa; 6 HAMP and 2 HK domains and 1 RR domain; transmembrane protein; highly conserved in *H. capsulatum* and *C. immitis*	Cell wall, virulence, dimorphism; in *H. capsulatum* expression of virulence factors, dimorphism, and sporulation	49
C. neoformans	Tco1p	1,383 aa; 7 HAMP domains; cytosolic protein	Mating; negative regulator of melanin and virulence; hypoxia adaptation	4, 21, 23
	Tco2p	1691 aa; 2 HK and 2 RR domains; cytosolic protein	Peroxide resistance	4
C. albicans	Sln1p	1,373 aa; single HK, HAMP, and RR domains; transmembrane protein	Osmosensor; partial virulence	48, 73
	Nik1p	1,081 aa; 5 HAMP and 2 HK domains and 1 RR domain; cytosolic protein	Morphogenesis; partial virulence	48, 59, 73
	Chk1p	2,471 aa; partial Ser/Thr kinase and GAF domains; cytosolic protein	Cell wall synthesis, quorum sensing, triazole resistance, virulence	10, 19, 73
C. lusitaniae	Sln1p	1,196 aa; single HK, HAMP, and RR domains; transmembrane protein	Morphogenesis, oxidative stress	16
	Nik1p	1,083 aa; 5 HAMP and 2 HK domains and 1 RR domain; cytosolic protein	Moderate sensitivity to oxidants	16
	Chk1p	2,397 aa; partial Ser/Thr kinase and GAF domains; cytosolic protein	Moderate sensitivity to oxidants	16
A. fumigatus	fos1p	1,096 aa; 2 HK domains, 1 coiled coil, and 1 RR domain; transmembrane protein	Virulence	22

[a] aa, amino acids; HAMP, hepcidin antimicrobial peptide; GAF, cyclic GMP phosphodiesterase/adenylcyclase/FhlA.

minimally used so energy is conserved. When stress signals are detected by cells (oxidants, high salt, etc.), the RR protein is not phosphorylated and is now able to activate the HOG1 MAPK pathway to adapt cells to stress (Fig. 2B). In the case of human pathogens, functions of this pathway compared to those of model fungi are expanded to regulate a number of other attributes such as virulence, host cell recognition, morphogenesis/dimorphism, survival in neutrophils, mating, and quorum sensing (see below). It should be mentioned that mutants lacking genes in the HOG1 MAPK pathway (*ssk2*, *pbs2*, *hog1*) are also oxidant sensitive and, at least in the case of the *hog1* mutant, avirulent (18).

DEFINING SUITABLE DRUG TARGETS

A putative drug target is defined by fulfilling several requirements. For example, the target must be (i) present in most if not all pathogenic fungi, (ii) absent in humans (if it is present, the drug may be designed to increase the specificity for the pathogen and therefore avoid toxicity to the host), (iii) relevant to the disease process, and (iv) such that a high-throughput assay to screen compounds can easily be developed. All four requirements are addressed below.

(i) The target should be present in most pathogenic fungi. Of the requirements mentioned above, the availability of whole-genome data sets of fungal pathogens has simplified the identification of broadly conserved orthologues (5, 7, 27, 43, 51). Thus, among pathogens of humans, two-component proteins are found in *C. albicans* (1, 2, 9–14, 17–19, 26, 35–38, 40, 41, 49, 60, 62, 75); *Candida lusitaniae* (16, 58); *Candida glabrata* (28); *Cryptococcus neoformans* (4, 21, 23, 72); *Aspergillus fumigatus* (22, 25, 39, 55, 74); and the endemic mycosis fungi, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Coccidioides immitis*/*Coccidioides posadasii*, and *Histoplasma capsulatum* (5, 27, 34, 50), as well as model fungi and plant pathogens. Data are summarized for human pathogens in Tables 1 and 2. Among fungi, the number of HK and RR proteins varies, but generalizations can be made, as in the following examples. (i) Yeasts such as *S. cerevisiae*, *C. albicans*, and *Schizosaccharomyces pombe* have fewer HK proteins than filamentous *Ascomycetes* like *Cochliobolus heterostrophus*, which has 21 HK proteins, and *Gibberella moniliformis*, which has 16 HK proteins (14, 32). The human pathogen *Aspergillus fumigatus* has at least 10 HKs (51). (ii) A phylogenetic analysis of the filamentous *Ascomycetes* revealed that the HKs fall into 11 major classes, but some of these classes are more often represented among plant pathogens than among saprobic fungi, suggesting a correlation between these HKs and the disease-causing ability of pathogens (14). (iii) Again, with the filamentous *Ascomycetes*, and for that matter with human pathogens, there are far fewer RR proteins than HKs per organism, suggesting that divergent signals are recognized by HKs and then transferred to common RR proteins. Based upon this information, it would appear that the RR proteins more likely would represent a suitable point of attack from the standpoint of drug development. However, in *C. albicans* (75) and *C. lusitaniae* (16) *sln1*, *chk1*, and *nik1* double and triple mutants are inviable, suggesting that a drug which targets these proteins may have similar effects. (iv) HK proteins are represented among fungi that cause the en-

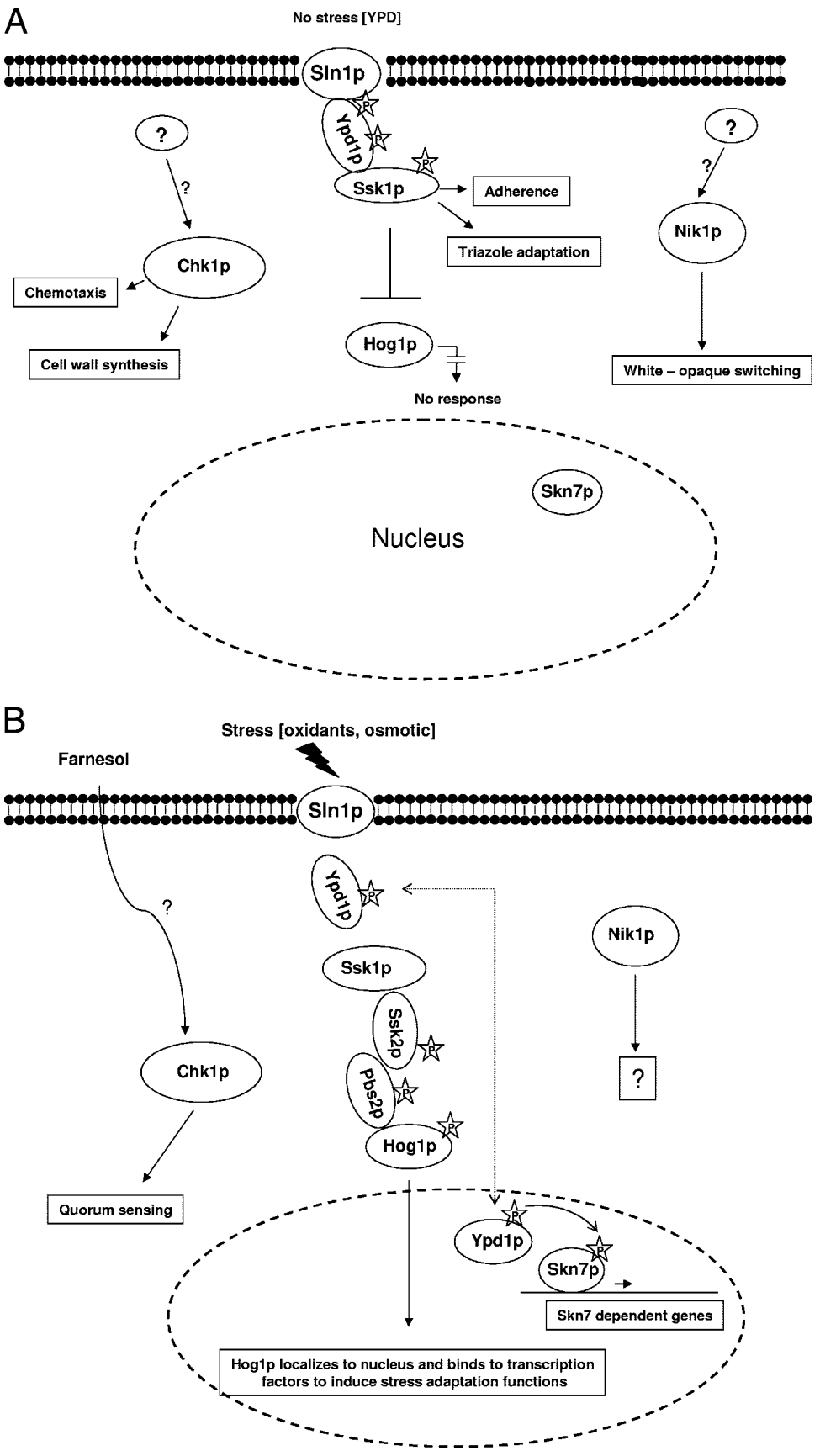
A
No stress [YPD]
Sln1p
Ypd1p
Ssk1p
Adherence
Triazole adaptation
?
?
Chk1p
Chemotaxis
Cell wall synthesis
Hog1p
No response
?
?
Nik1p
White – opaque switching
Skn7p
Nucleus
B
Stress [oxidants, osmotic]
Farnesol
Sln1p
Ypd1p
Ssk1p
Ssk2p
Pbs2p
Hog1p
?
Chk1p
Quorum sensing
Nik1p
?
Ypd1p
Skn7p
Skn7 dependent genes
Hog1p localizes to nucleus and binds to transcription factors to induce stress adaptation functions

TABLE 2. Selected RR proteins of medically important fungal pathogens

Organism	RR	Protein features[a]	Functions	Reference(s)
C. neoformans	Ssk1p	1,352 aa; single RR domain; cytosolic protein	Stress adaptation, capsule and melanin production	4
	Skn7p	1,027 aa; single HSF and RR domains; nuclear protein	Stress adaptation, melanin production, virulence	4, 71
C. albicans	Ssk1p	674 aa; single RR domain; cytosolic protein	Adherence, triazole resistance, oxidative stress adaptation, virulence	12, 17, 19, 47
	Skn7p	559 aa, single HSF, coiled-coil, and RR domains; nuclear protein	Oxidative stress	18
C. lusitaniae	Ssk1p	614 aa; single RR domain; cytosolic protein	Osmotic stress and pseudohyphal growth	57
	Skn7p	479 aa; single HSF and RR domains; nuclear protein	Oxidative stress adaptation	57
A. fumigatus	Skn7p	597 aa; single HSF and RR domains; nuclear protein	Oxidative stress; conidia are more readily killed by human PMNs[b]	39

[a] aa, amino acids; HSF, heat shock factor.
[b] PMNs, polymorphonuclear leukocytes.

demic mycoses, such as *B. dermatitidis*. (v) The pathogenic basidiomycetous fungi are also represented, as *C. neoformans* has seven HKs (Tco1 to -7), one Hpt, and two RR proteins, Ssk1 and Skn7 (4, 21, 23, 72). This brief summary demonstrates that the proteins (both HK and RR) are conserved across several fungal pathogens infecting humans. Importantly, functional annotation has been assigned to most of these two-component genes by evaluating gene-deleted mutants of several of the fungal pathogens infecting humans (see below). The reader is directed to several reviews that describe construction of fungal pathogen mutants (8, 50, 53, 56, 68, 69).

(ii) The target should be absent in humans. The two-component proteins were originally described in bacteria, lower eukaryotes, and plant cells but were thought to be absent in mammalian cells. HKs have escaped detection in mammalian cells in part because of the acid lability of histidine phosphorylation, although that characteristic is constant among all HK proteins. Nevertheless, the phosphorylation of a histidine residue of the K^+ channel protein KCa3.1 has recently been identified from mammalian cells (63, 64). However, the similarity of this protein to fungal HKs such as Sln1p, determined by Clustal W analysis, is minimal. It is also of importance that, for the mammalian system, histidine phosphotransfer activates downstream events, while in fungi, phosphorylation of the downstream Hpt and RR proteins inhibits MAPK activity and transcriptional regulation of structural genes. There, anti-HK drugs should have minimal reactivity with proteins such as KCa3.1. As for the RR proteins of fungi, a search of the literature has not revealed their presence in mammalian cells. Given that background, two-component HK and RR (especially) proteins would appear to qualify as specific targets of bacteria and fungi.

(iii) The target(s) must be relevant to the disease process. In the following section, we address the relationship of these proteins to the disease process in fungi that cause endemic mycoses (*B. dermatitidis*, *H. capsulatum*), as well as *C. neoformans*, *C. albicans* (and other *Candida* species), and *A. fumigatus*; data are summarized in Tables 1 and 2. The proteins that are listed in these tables are only those studied and are not meant to include all HK and RR proteins in fungi pathogenic to humans.

***(a)* The endemic mycoses.** This group of human pathogens includes *B. dermatitidis* and *H. capsulatum*, for which functional data have been reported (50). In addition, the genomes of *C. immitis*/*C. posadasii* and *P. brasiliensis* (Broad Institute; released in 2006) also have homologues of HK- and RR-encoding genes (5, 27), although functional analysis of the last two pathogens has not been published. Following inhalation of spores from the environment or a temperature shift from 25°C to 37°C in vitro, these organisms change their morphology, and this growth transition is referred to as temperature-dependent dimorphism. In the case of *B. dermatitidis*, *H. capsulatum*, and *P. brasiliensis*, growth at 25°C is filamentous (mold), while at 37°C the organism converts to a yeast growth form. Yeast-

FIG. 2. Signal pathways that include each of the HKs and RRs of *C. albicans*. (A) The HOG1 MAPK kinase pathway and the upstream two-component phosphotransfer proteins, Sln1p, Ypd1p, and Ssk1p. In the absence of stress, phosphotransfer reactions occur on these proteins and involve the following chain of residues H→D→H→D (top). Phosphorylated Ssk1p is unable to activate the MAPK kinase kinase Ssk2p, and the HOG1 MAPK pathway is inactive. Also shown are the HKs Chk1p and Nik1p. Both are positioned as cytosolic proteins; environmental cues are incompletely understood for these proteins, but functions of each are shown. The Skn7p RR protein is depicted in its nuclear location. (B) During stress, the phosphotransfer depicted in panel A does not occur. Ssk1p then activates the phosphorylation of Ssk2p and subsequent phosphorylation of Pbs2 and Hog1p (SsK2→Pbs2→Hog1). Phosphorylated Hog1p translocates to the nucleus and activates genes required for the adaptive response. The quorum-sensing (QS) function of the Chk1p HK following its interaction with the QS inducer farnesol is also shown. In *S. cerevisiae*, during stress, Ypd1p translocates to the nucleus and phosphorylates Skn7p, resulting in new gene transcription and adaptation to stress (44). Phosphotransfer is indicated as stars containing a P (phosphate).

specific genes have been identified, one being *BAD1* (*Blastomyces ad*hesion), but the regulation of these genes (like *BAD1*) and the yeast morphology in fact is not completely understood. To this end, insertion mutants were isolated to identify genes that regulated dimorphism (50). Phenotypically, presumed mutant colonies demonstrated cell wall defects, decreased *BAD1* transcription, and an inhibition of hyphal to yeast morphogenesis. Complementation of the defects was achieved with the DNA sequence (ORFA) associated with the insertion mutant. The ORFA sequence matched that of *SLN1*, encoding HK of *S. cerevisiae*, and also those of *H. capsulatum* and *C. immitis* genes and was named *DRK1* (*d*imorphism-*r*egulating H*K*). RNA interference was then used to construct *drk1* mutants of both *B. dermatitidis* and *H. capsulatum*, and the phenotypes of those mutants included altered growth at 37°C (pseudohyphae, not yeasts) and significantly reduced expression of virulence genes (Table 1). Thus, the product of *DRK1* (an HK) would seem to qualify as a global regulator of dimorphism and virulence factor expression in these two fungi.

(b) Cryptococcus neoformans. The functions of two-component signaling proteins in *C. neoformans* have been reported (4, 21, 23, 72). *C. neoformans* has seven hybrid HKs (Tco1p to -7p), two RR proteins (Ssk1p and Skn7p), and a single phosphohistidine intermediate protein (Ypd1p). Bahn et al. (4) demonstrated their role in stress adaptation, antifungal sensitivity, virulence factor regulation, and sexual reproduction. Unlike those of many other fungi, all seven HKs in *C. neoformans* lack a transmembrane domain, suggesting that they are all cytosolic proteins (Table 1; Fig. 1A). One of the HKs (Tco2) is unique in that it has two HK and two RR (receiver) domains, a feature that has not been reported among any known hybrid HKs of pathogenic fungi.

As in *C. albicans*, the Ssk1 RR of *C. neoformans* regulates the Hog1 MAPK pathway. An *ssk1* mutant had phenotypes identical to those of *pbs2* and *hog1* mutants, as expected if Ssk1p is upstream of the HOG1 MAPK pathway. The *ssk1* mutant was hypersensitive to oxidative stress, osmotic stress, and UV irradiation but was resistant to antifungal drug fludioxonil (Table 2). Phosphorylation of Hog1p in response to fludioxonil and methylglyoxal was not observed in the *ssk1* mutant, suggesting that a functional Ssk1 protein is needed to activate Hog1p. Disruption of *SSK1* leads to a significant increase in capsule and melanin production, two major virulence factors of *C. neoformans*. The role of Ssk1p in virulence was not investigated by these authors. The second response regulator, Skn7p, appeared to function in a Hog1-independent signaling pathway because most of the phenotypes it produces do not match those of the *ssk1*, *pbs2*, and *hog1* mutants (72). The *skn7* mutant was sensitive to oxidants and Na^+ ions and resistant to fludioxonil, had hyperactive melanin production but not capsule synthesis, and was attenuated in a mouse model compared to wild-type cells (Table 2). Hog1 was phosphorylated in the *skn7* mutant in response to osmotic stress (1 M NaCl), similar to what is found for wild-type cells.

Bahn et al. (4) reported that the Ypd1 Hpt protein may be essential for cell viability because they could not construct a deletion strain. Also reported was that six HK genes (the Tco1, -2, -3, -4, -5, and -7 genes), but not the Tco6 gene, could be disrupted, suggesting that Tco6 is essential for viability. Among all of the seven HKs, Tco1 and Tco2 produce Hog1-related phenotypes. Tco1 plays a role in conferring sensitivity to fludioxonil and methylglyoxal, regulation of melanin biosynthesis, and sexual reproduction. Tco2 on the other hand is responsible for osmotic and oxidative stress and drug sensitivity.

These authors also investigated the role of Tco1 and -2 in the virulence of *C. neoformans*. The Tco1 mutant was attenuated compared to wild-type cells in a mouse model of cryptococcal meningitis, while the Tco2 deletion strain was as virulent as the wild type.

(c) Candida albicans. There are three HKs, two RRs, and a single Hpt protein in this organism and orthologues in *C. lusitaniae* and *C. glabrata* (16, 28, 35, 58) (Fig. 1). Of the upstream two-component proteins that regulate the HOG1 MAPK pathway, functions for Sln1p (HK) and Ssk1p (RR) have been assigned on the basis of gene-disrupted mutants, while a Ypd1p deletion mutant has not been constructed. *C. albicans sln1*, *chk1*, *nik1*, and *ssk1*, but not *skn7*, deletion mutants are attenuated in a murine model of hematogenously disseminated candidiasis (10, 12, 48, 60, 75). Phenotypic changes in mutants vary according to the specific gene deletion, but all mutants have impaired patterns of morphogenesis. Other phenotypes include osmo (*sln1*, *nik1*)- and oxidant sensitivity and reduced survival in human neutrophils (*ssk1*, *chk1*) (26, 40, 49, 65) (Tables 1 and 2). The *nik1* mutant is also partially reduced in its ability to display the opaque-white switch phenotype, which is required for mating and virulence (62). In *C. lusitaniae*, the functions of SLN1 include morphogenesis and oxidant adaptation (58).

Most of the remaining discussion on the *C. albicans* proteins will focus upon Chk1p and Ssk1p. The phenotypes in a mutant lacking CHK1 suggest an altered cell wall (6, 36, 41), including (i) reduced adherence to human esophageal cells in vitro, (ii) flocculation of cells in M199 medium as opposed to wild-type cells and a gene-reconstituted strain that does not flocculate (11), (iii) changes in the ratio of β-1,3- to β-1,6-glucans, with a decrease in the former and an increase in the latter, resulting in an increase in sensitivity to the cell wall inhibitor Congo red in vitro, and (iv) oligosaccharide truncation of wall proteins, suggesting that the mutant is unable to properly glycosylate protein or synthesize the full-length mannosyl oligosaccharide (36). Also, the chk1 mutant is refractory to quorum sensing (38).

The absence of the Ssk1p RR of *C. albicans* in gene-deleted strains results in (i) oxidant sensitivity in vitro and greater killing by human neutrophils (17, 26), (ii) reduced adherence to human esophageal tissues and endothelial cells in vitro (41), and (iii) attenuated virulence in a murine model of hematogenous dissemination (12) and reduced colonization in a rat vaginitis model (48) (Table 2).

More recently, the role of two-component signal proteins and MAPK pathways in drug sensitivity was studied (19). These studies were initiated because of the prominent role that two-component signaling plays in drug resistance in the bacterial pathogens *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Escherichia coli*, although this is only a partial list of bacterial pathogens that use two-component signaling in their adaptation to antibacterial drugs (29, 46, 47, 52). To investigate the activity of two-component proteins in antifungal drug sensitivity/resistance in *C. albicans*, MIC assays were performed with a number of antifungal drugs and general cell

inhibitors. For both the *chk1* and *ssk1* mutants, no changes in sensitivity to amphotericin B, flucytosine, caspofungin, or imidizoles such as ketoconazole and miconazole were observed. However, quite strikingly, the response of the *ssk1* and *chk1* mutants was a hypersensitivity to the triazoles voriconazole and fluconazole, approaching a 50- to 300-fold increase in drug sensitivity. While other triazoles have not been assayed similarly, current data indicate that the mutations are specific for triazole drugs. But what is responsible for this hypersensitivity? To address this question, uptake of [^{3}H]fluconazole was measured and found to increase by twofold in the *ssk1* mutant compared to the parental strain and a gene-reconstituted strain (19). Of other two-component protein mutants, the *chk1* mutant also had increased uptake of fluconazole. Cell accumulation of fluconazole is due to both uptake and efflux of the drug; however, reverse transcription-PCR comparisons indicated little in the way of changes in transcription of known efflux pump genes of the *ssk1* mutant versus parental cells. Concomitantly, the *ssk1* and *chk1* mutants are also killed to a greater extent than parental cells (fluconazole, 5-fold increase in killing; voriconazole, 10-fold increase in killing). Thus, in addition to their prominent roles in virulence, both the Ssk1p RR and Chk1p HKs regulate net cell accumulation of at least fluconazole, and their deletion increases the fungicidal activity of normally fungistatic drugs.

(d) Aspergillus fumigatus. Several two-component signal proteins produced by *A. fumigatus* (fos1, skn7, tscB), as well as a HOG1 MAPK homologue (saKA), have been studied (Tables 1 and 2). A *fos1* deletion strain was constructed and evaluated phenotypcially (22). fos1 is a homologue of the NIK1 HK described above. The mutant was also found to be attenuated in virulence compared to the wild-type parental strain in an intravenous-infection murine model of IA but was not tested in an intranasal model of IA, so the role of this protein in the natural history of IA remains to be established. The Skn7 RR was shown sensitive to hydrogen peroxide, and conidia were more readily killed by human neutrophils than wild-type conidia, but the deletion mutant was as virulent as wild-type cells in a murine model of pulmonary aspergillosis (39). As for studies of other HK proteins, *tscB* (*SLN1* homologue) in *A. fumigatus* was dispensable for most cell functions (25). The Hog1 homologue of *A. fumigatus* (SakA) is likewise peroxide sensitive and involved in nitrogen sensing and germination of conidia, but its role in virulence is unknown (25, 74).

(iv) High-throughput assays for new drugs should be facile. The most direct approach to identify new drug targets is to screen a library of fungal mutants against compound libraries. A desirable outcome of such a screen is a compound that has reasonable activity against a wild-type strain (compared to current antifungals) but has increased activity inhibitory to an HK or RR mutant. In diploid pathogens such as *C. albicans*, strains lacking one or both functional alleles have been constructed using several approaches such as gene replacement and conditional expression (42, 57) and haploinsufficiency (HI) (66, 73; see references 8 and 68 for reviews) to identify growth-essential genes and screen for drug-target pairs against compound libraries. A similar approach has been used to identify growth-essential genes in *A. fumigatus* (31). HI refers to a growth phenotype that is associated with a loss of an allele in a diploid strain achieved through conditional promoter replacement or allele deletion. In a strain lacking a copy of a functionally validated or inferred gene, a mechanism of action (MOA) may be assigned to a specific compound to which that mutant is sensitive. In *C. albicans*, HI has been used to identify those genes associated with morphogenesis functions (66). Also, Xu et al. used HI to identify on a genomic scale hypersensitivity to antifungal drugs in *C. albicans* (73). In brief, their assay, referred to as the *Candida* fitness test, utilized strains in which an allele was replaced with a cassette consisting of a selection marker flanked by homologous sequences of randomly selected genes and, in turn, unique up and down tags so that each deleted allele could be bar coded by PCR. About 2,800 heterozygous deletion strains were constructed with alleles chosen by their growth essentiality and broad representation in other fungi. For screening, mutant pools were incubated with compound libraries and strain abundance was measured by microarrays that differentiated sensitive heterozygotes from strains with wild-type levels of resistance. As proof of principle, strains with known sensitivities to fluconazole and AmpB (among others) were evaluated similarly. Among other observations, the authors used HI to identify compounds that specifically affected microtubule functions.

Especially attractive, therefore, is the use of a library of HI strains, such as two-component gene mutants from *C. albicans*, to screen for new compounds. In theory, therefore, whole-cell assays of wild-type strains versus heterozygotes (HI) or gene off/gene on strains could be an easy way to evaluate new compound libraries versus the use of a purified protein target. As just stated, the desired end point of such assays would be a compound(s) to which HK or RR heterozygote strains have increased sensitivity compared to wild-type strains; wild-type strains should exhibit a sensitivity that is comparable to that to known antifungals (19). Since the *C. albicans chk1* and *ssk1* mutants are hypersensitive and killed by the triazoles fluconazole and voriconazole, another approach may be to identify compounds that inhibit these proteins that would also interact synergistically with triazoles that are notoriously fungistatic (19).

CONCLUSIONS

While triazoles and caspofungin (β-1,3-glucan inhibitor) have been added to the list of antifungal drugs, problems with drug resistance (candidiasis, triazoles) and less (IA, caspofungin) or no (cryptococcosis, caspofungin) efficacy have made searches for new drugs a requirement. With the triazoles, most likely their use has changed the frequency among species, reducing the frequency of candidiasis due to *C. albicans* relative to that of candidiasis due to *Candida* species other than *C. albicans*. The identification of broadly conserved, growth-essential genes has been achieved in part by comparing genome databases of human fungal pathogens to that of *S. cerevisiae* (5, 7, 27, 44). Conventional thinking is that some of these genes represent desirable drug targets. We hypothesize that compounds that target regulatory proteins that are broadly conserved across pathogenic fungi, that are not found in humans, and that have been demonstrated to be important in the regulation of virulence offer potential as targets for new drug discovery. In this regard, the critical role of two-component signal proteins in the disease process has been validated for

several fungi pathogenic to humans. We would hope that validation of these proteins in pathogens such as *C. immitis*/*C. posadasii* and *P. brasiliensis* is forthcoming. Screens of compound libraries against two-component mutants of human pathogens constructed to exhibit an HI should lead to the identification of compound-target pairs. For further development as a lead compound, the sensitivity of the mutant to that compound should be augmented significantly compared to the sensitivity of a wild-type strain and the wild-type strain should have a sensitivity that parallels its sensitivity to established antifungals. Thus, in the case of two-component proteins, HI can yield correlates of a phenotype (compound sensitivity) due to a mutation in an HK or RR. As shown by others (see "High-throughput assays for new drugs should be facile" above), HI is useful in defining a MOA of the compound if sensitivity is observed; in the case of two-component proteins, the MOA would already be at least partially established.

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