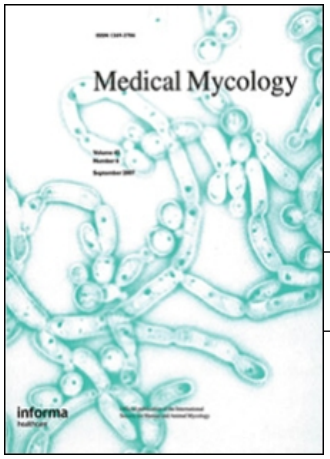

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Fungal β(1,3)-D-glucan synthesis

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The polysaccharide β(1,3)-D-glucan is a component of the cell wall of many fungi. Synthesis of the linear polymer is catalysed by UDP-glucose β(1,3)-D-glucan β(3)-D-glucosyltransferase. Because this enzyme has a key role in fungal cell-wall synthesis, and because many organisms that are responsible for human mycoses, including *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*, produce walls that are rich in β(1,3)-glucan, it has been and remains the focus of intensive study. From early characterization of the enzymatic activity in *Saccharomyces cerevisiae*, advances have been made in purification of the enzyme, identification of essential subunits and description of regulatory circuitry that controls expression and localization of different components of the multisubunit enzyme complex. Progress in each of these areas has been enhanced dramatically by the availability of specific inhibitors of the enzymatic reaction that produces β(1,3)-glucan. These natural product inhibitors have utility both as tools to dissect the biology of β(1,3)-glucan synthase and as sources for development of semisynthetic derivatives with clinical utility in treatment of human fungal disease. This review will focus on the biochemistry, genetics and regulation of the enzyme.

Keywords β(1,3)-glucan, *FKS* genes, echinocandin

Biochemistry

In 1980, Enrico Cabib and his colleagues described studies on β(1,3)-glucan synthetase from *S. cerevisiae* [1,2]. A pair of landmark papers reported the preparation and properties of the enzyme and outlined a role for guanosine and adenosine nucleotides in enzyme activation. The catalytic activity was measured using crude membranes as the source of enzyme; maximal activity was obtained in reaction mixtures buffered to pH 8.0 and containing UDP-glucose as substrate, GTP, glycerol and bovine serum albumin. The reaction product was confirmed as authentic β(1,3)-glucan by virtue of its solubility in alkali, insolubility in water or dilute acid, susceptibility to digestion with purified β(1,3)-glucanase but insensitivity to α- and β-amylase, complete resistance to periodate oxidation (which cleaves 1,2-dihydroxy linkages absent in linear β(1,3)-glucan; [3]), and conversion to glucose upon complete acid hydrolysis. The polysaccharide produced in the reaction was estimated to be 60–80 glucose residues long. A working model based on these results proposed that an ATP-dependent phosphorylation event might work in concert with a guanine nucleotide to regulate this component of cell-wall synthesis during the yeast cell cycle. Continued study of the biology of β(1,3)-glucan synthase from *S. cerevisiae* and other fungi has only served to underscore the insight of this original hypothesis.

Many of the themes uncovered in these and other early studies [4] are echoed in characterization of enzymatic activity from other organisms. β(1,3)-glucan synthase has been studied in filamentous ascomycetes such as *Neurospora crassa* [5], *Aspergillus nidulans* [6], and *Aspergillus fumigatus* [7,8], in non-filamentous ascomycetes such as *Schizosaccharomyces pombe* [9], in deuteromycetes such as the dimorphic fungus *Candida albicans* [10], in basidiomycetes such as *Cryptococcus neoformans* [11], and even in oomycetes such as *Phytophthora* spp., which are not true fungi [12, 13]. Among these diverse sources, all enzymes are membrane-associated complexes that use UDP-glucose as a

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substrate (the K_M is typically 1–5 mM in crude enzyme preparations) and produce a linear polysaccharide. Several studies have verified that the reaction is processive, adding one mole of glucose for every mole of UDP-glucose hydrolysed to produce chains of increasing length ([1,14]; J. Williamson, unpublished result). This distinguishes β(1,3)-glucan synthase from non-processive glycosyltransferases, which transfer sugar residues (either mono- or oligosaccharides) to acceptor molecules that include proteins, lipids or other sugars [15]. Specific details about product length, including factors that control the degree of polymerization both in cell-free extracts or in whole cells, have not been described for any fungal enzyme. Finally, although it is clear that polymer length is extended as the β(1,3)-glucan synthase reaction proceeds *in vitro*, the issue of whether or not synthesis can be initiated *de novo*, or requires a glucan acceptor, remains unresolved [14,16].

To date, there has not been a report of purification of fungal glucan synthase to homogeneity. Major advances towards identifying subunits of the enzyme complex have come from fractionation, solubilization and product entrapment of the activity. By treating samples with detergent and salt, Kang and Cabib [17] described dissociation of the enzyme in crude membranes derived from either *Hansenula anomala* or *N. crassa* into a soluble fraction and a membrane-associated fraction. Neither of the fractions exhibited significant activity alone, but reconstitution of a soluble fraction with a membrane fraction, either from the same organism or as a heterologous mixture, resulted in an active enzyme. Within each fraction, the proteinaceous component(s) required for restoration of activity could be inactivated by heat or trypsin digestion, but protection from heat inactivation could be conferred to the membrane-associated fractions by UDP-glucose, and to the soluble fractions by GTP-γ-S. An equivalent fractionation scheme has yielded similar results with enzyme derived from *S. cerevisiae* [18,19], *S. pombe* [20], *C. albicans* [21], or *A. nidulans* [6]. The use of Tergitol NP-40 in these studies has been expanded upon and other detergents have been found useful for solubilizing β(1,3)-glucan synthase from crude membranes. Extraction with CHAPS plus octyl glucoside, CHAPS alone, Brij-35 or polyoxyethylene ether has yielded soluble activity from membrane preparations of *N. crassa* [22], *A. fumigatus* [7], *S. cerevisiae* [23] and *C. albicans* [24], respectively. However, neither fractionation nor solubilization has provided dramatic purification of the enzyme, and traditional methods such as ammonium sulphate precipitation [25] and chromatography [7] have been of limited utility. A key step forward has been the use of product entrapment, first described for chitin synthase from *S. cerevisiae* [26], to enrich for subunits of the β(1,3)-glucan synthase complex [25]. The polysaccharide produced during *in vitro* synthesis becomes insoluble as the degree of polymerization increases, and enzyme remains associated with this nascent glucan. The enzyme–product complex can be harvested from reaction mixtures by centrifugation, washing and solubilization of the enzyme, followed by another round of product entrapment. This technique has been exploited for purification of β(1,3)-glucan synthase from *N. crassa* [25], *A. nidulans* [6], *A. fumigatus* [27], *S. cerevisiae* [28] and *C. albicans* [29], and the specific activity of enzyme prepared by this method is the highest reported to date. Although not pure, enzyme prepared in this manner may be amenable to a proteomic-based analysis to confirm the identity of specific subunits which have been identified by other means (see below), or lead to identification of new ones.

Photoaffinity probes have been used in an alternate strategy to characterize subunits of β(1,3)-glucan synthase [7,22,23]. Partially purified enzyme from either *S. cerevisiae*, *N. crassa*, or *A. fumigatus* has been photolabelled with either 5-azido-[^{32}P]UDP-glucose or 8-azido-GTP, and the spectrum of photoproducts has been analysed. As expected, purity of the sample used for photolysis has a significant effect on the photoproduct profile. With 8-azido-GTP, initial studies using particulate *S. cerevisiae* glucan synthase [23] failed to identify the 20-kDa GTP-binding polypeptide that was found when a chromatographically purified fraction was photolabelled [30]. This protein, Rho1p, is a key regulatory component of β(1,3)-glucan synthase and a member of the enzyme complex [21]. With 5-azido-[^{32}P]UDP-glucose, a protein of 165 kDa from *N. crassa* [22] and proteins of 31, 50 and 115 kDa from *A. fumigatus* [7] have been labelled. The samples used for labelling were partially purified by either product entrapment (*N. crassa*) or solubilization and chromatography on a Bio-gel P30 column (*A. fumigatus*). Although there have been no subsequent reports to clarify the role of these polypeptides in the catalytic complex, the 165-kDa *N. crassa* protein is enriched during purification, and the photocrosslinking event can be competed with UDP-glucose [22]. As with photolysis using 8-azido-GTP, identification of the bona fide catalytic subunit of the enzyme by this method may require further purification.

Inhibitors of β(1,3)-glucan synthase

A report describing inhibition of β(1,3)-glucan synthesis in cell-free extracts from *S. cerevisiae* and *C. albicans* was published in 1982 [31]. The impetus to suspect this

enzyme as a target for several natural products with antifungal activity, including aculeacin A [32], echinocandin B [33] or papulacandin B [33], came from work on whole cells and spheroplasts. Cells exposed to these compounds showed morphological changes such as swelling and release of intracellular contents, and metabolic labelling revealed preferential inhibition of incorporation of radiolabelled glucose into cell-wall polysaccharides [32]. When the wall components were fractionated, the alkali-insoluble fraction, which is enriched in glucan rather than mannan, was affected specifically [33, 34]. Direct measurement of chitin or mannan biosynthetic activity in the presence of aculeacin A revealed complete lack of inhibition at concentrations that inhibited β(1,3)-glucan synthase activity by as much as 70% [35]. The enzymes used in these experiments were derived from organisms, including *Geotrichum lactis* [36], *C. albicans* [37], *S. cerevisiae* [31], *N. crassa* [38], *H. anomola*, *Cryptococcus laurentii* and *Schizophyllum commune* [39]. Three themes emerged from these early studies: First, inhibition of enzymatic activity was not competitive with the UDP-glucose substrate; second, reactions were inhibited to 70 or 80% of control, but not to completion; and third, susceptibility to inhibition is conserved across several fungal genera.

There are three general structural classes that define known natural product inhibitors of β(1,3)-glucan synthesis. The first class, the lipopeptides, comprises cyclic hexapeptides with an *N*-linked fatty-acyl side chain [40]. Included in this group are the aculeacins, the echinocandins, the pneumocandins, mulundocandin and FR901379 [41]. The second family of inhibitors are the glycolipid papulacandins, which consist of a modified disaccharide linked to two fatty-acyl chains [42]. The third group, including the recently discovered acidic terpenoids, are represented by enfumafungin, ascosteroside, arundifungin and ergokonin A [34]. Several reviews provide insight into the diversity of compounds that have been discovered in the lipopeptide and papulacandin classes [43,44,41,45]; at the moment, the acidic terpenoid group consists of only a few members.

Discovery of specific inhibitors of β(1,3)-glucan synthesis, combined with the appeal of a potentially broad spectrum, fungal-specific target has fuelled interest from several pharmaceutical companies who are seeking novel treatments for human mycoses. Several natural lipopeptides that exhibited promising attributes, such as activity in animal models of fungal infection [46,47], have served as templates for medicinal chemistry efforts. Key issues such as improvement of water solubility [48], expansion of antifungal spectrum to include other significant human pathogens beyond *Candida* spp. [49], optimization of desirable pharmacokinetic properties [50], and elimination or reduction of off-target activity have been addressed [44,45]. These efforts have succeeded in producing one currently licensed antifungal agent [51], with two others in late-stage clinical development [52,53]. Caspofungin acetate (Cancidas®) from Merck & Co. has been approved by the US Food and Drug Administration and is indicated for the treatment of invasive aspergillosis in patients who are intolerant of or refractory to existing therapies [51]. This semisynthetic derivative of pneumocandin B_0 has shown excellent safety and tolerability in clinical use to date. In phase II clinical studies, the efficacy and safety of caspofungin was compared to that of fluconazole (a licensed antifungal antibiotic which targets is lanosterol demethylase) in adult patients with *Candida* esophagitis. Both compounds were generally well-tolerated and demonstrated similar response rates, with ~95% of patients experiencing resolution of symptoms by the end of therapy (median time less than five days; A. Villanueva, E. Gottuzzo, A. Arathoon, L.M. Noriega, N. Kartsonis, R. Lupinacci, J. Smietana, R.S. Berman, M.J. DiNubile, and C.A. Sable, unpublished results). Micafungin (Fujisawa Pharmaceutical Co.) and anidulafungin (V-echinocandin; Versicor) have also demonstrated efficacy in clinical trials [54].

Chemical derivatization of lipopeptides has provided excellent new tools for studying β(1,3)-glucan synthesis. Modification of LY303366 (anidulafungin) by scientists at Eli Lilly & Co. created an ^{125}I-labelled 4-azidobenzamidine photoaffinity probe, with potency against *C. albicans*, that was essentially unchanged from the parent compound [55]. This substituted echinocandin was used to photolabel crude microsomes containing active β(1,3)-glucan synthase. Proteins with molecular weights of 40 and 18 kDa were strongly labelled on autoradiograms of photolysed mixtures that had been separated by SDS–PAGE. The interaction between the compound and these proteins seems to be specific, as LY303366 competed in the photolabelling reaction, but addition of the separated components that make up the photoaffinity probe (the cyclic peptide nucleus and/or the terphenyl side chain) did not. Through partial proteolysis of the 40-kDa protein, the authors generated peptide sequences and used them to identify homologous proteins in the *S. cerevisiae* proteome. Unfortunately, little is known about these homologues, and no further characterization of the photoproducts has been published. At Merck, aminoethyl ether substitutions at two positions on the pneumocandin B_0 nucleus [48] produced L-733560, a compound with vastly improved potency against *S. cerevisiae* whole cells and β(1,3)-glucan synthetic activity derived from them [18]. In a genetic selection using L-733560, mutants were isolated that

pointed to a gene encoding a subunit of the enzyme complex. Work on this gene, both in *S. cerevisiae* and in other fungi, has provided a major advance in understanding the genetics of β(1,3)-glucan synthase.

Genetics

Surprisingly, it was research efforts focused on yeast as a model system for studying the Ca^{2+}–calmodulin-dependent phosphoprotein phosphatase calcineurin, and the mode of action of immunosuppressive compounds such as tacrolimus (FK506) and cyclosporin A (CsA) that helped to uncover genes encoding subunits of β(1,3)-glucan synthase. In 1993, an *S. cerevisiae* mutation that conferred calcineurin-dependent growth and hypersensitivity to tacrolimus was described, and the allele was designated *fks1-1* (for FK506-supersensitive) [56]. Simultaneous efforts from at least three laboratories studying calcineurin led to cloning and sequencing of the wild-type version of the gene, which was designated *FKS1* [57,58] or *CND1* [59]. Meanwhile, research on yeast cell wall biosynthesis led others to clone and sequence a gene which: first, complemented the Calcofluor White (CFW) hypersensitivity of mutant *cwh53-1* (Calcofluor White hypersensitive; CFW binds to chitin microfibrils and has a disordering effect on *S. cerevisiae* cell walls; [60]); second, encoded a protein that co-purified with β(1,3)-glucan synthase during product entrapment of the activity (*GSC1*; *g*lucan synthase of *S. cerevisiae* [62,28]; third, partially restored susceptibility to papulacandin B in strains which had been mutagenized and selected for resistance to this inhibitor (*pbr1-1* through *pbr1-8*; papulacandin B resistance; [20]); and fourth, complemented hypersensitivity to the chitin synthesis inhibitor Nikkomycin Z, which was found to be concomitant with spontaneous resistance to the echinocandin L-733560 in a single mutant (*etg1-4*) among several identified in a screen (*etg1-1* through *etg1-4*; *e*chinocandin *t*arget *g*ene; [18, 61]). That these two lines of research had identified the same gene has been formally verified [57]. Accordingly, the gene which complemented changes in susceptibility to inhibitors of cell-wall synthesis also reversed hypersensitivity of the *fks1-1* mutant to tacrolimus and cyclosporin A. Loss-of-function mutations in this gene made cells hypersensitive to immunosuppressants and caused about a sixfold reduction in β(1,3)-glucan synthesis activity. It became clear that the genes cloned and sequenced separately as *CND1*, *CWH53*, *ETG1*, *FKS1*, *GSC1* and *PBR1* were in fact the same.

How can mutations in a gene presumed to encode a subunit of β(1,3)-glucan synthase confer such diverse phenotypes as resistance to echinocandins, and hypersensitivity to nikkomycin Z or tacrolimus? One answer came from characterization of a second *S. cerevisiae* gene, *FKS2*, which encodes a protein with significant homology (87% identity) to the protein product of *FKS1* [63]. *S. cerevisiae* cells with a loss-of-function mutation in either gene are viable, but simultaneous disruption of *FKS1* and *FKS2* is lethal. The proteins Fks1p and Fks2p are considered to be alternate subunits of the β(1,3)-glucan-synthase enzyme complex with an essential overlapping function. When regulation of *FKS2* was studied, it was discovered that transcription is induced by Ca^{2+} in a calcineurin-dependent manner, and inhibited by tacrolimus. Because calcineurin is known to be the target of tacrolimus and CsA, it follows that cells with null alleles in *FKS1*, such as *fks1-1*, must exhibit hypersensitivity to tacrolimus or CsA because they rely on Fks2p for viability. There is no evidence for direct interaction between these immunosuppressants and either of the Fks proteins. As for the Nikkomycin Z-hypersensitive, echinocandin-resistant *etg1-4* mutant ([61] renamed *fks1-4*), the protein product Fks1-4p seems to be only partly functional, resulting in a cell wall that is deficient in β(1,3)-glucan (C.M. Douglas, S.A. Parent, W. Li, G. Chrebet, J.A. Marrinan, and M.B. Kurtz, unpublished results). Several studies have demonstrated that mutations that alter the composition of the wall induce compensatory changes in *S. cerevisiae*, including elevated levels of chitin [64,65,66,67; see review by Popolo *et al.* in this issue). The hypersensitivity to Nikkomycin Z, a chitin synthesis inhibitor, is likely to be a consequence of this increased dependency on chitin. The *fks1-4* mutant is unique among the L-733,560-resistant strains isolated at Merck [18,61]; each of the other *S. cerevisiae* mutations in *FKS1* confers both whole-cell and enzyme resistance to echinocandins without an effect on the specific activity of β(1,3)-glucan synthase, the K_M for UDP-glucose, or whole-cell susceptibility to inhibitors that affect other targets (e.g. polyenes, azoles, etc.). Later in this review, specific, distinct substitutions that are necessary and sufficient for the phenotypes associated with the echinocandin-resistance mutations (*etg1-1* and *etg1-3*), as well as the null allele *fks1-1*, are discussed.

The *FKS* genes are reasonably well conserved across a number of fungal genera [11]. Besides *S. cerevisiae*, homologues of *FKS1* have been found in a diverse collection of organisms (Table 1), including several that cause disease in humans. Hydropathy analysis of the large (>200 kDa) protein encoded by all *FKS* family members predicts a localization within the plasma membrane, with as many as 16 transmembrane helices. A central hydrophilic domain of about 580 amino acids displays a remarkable degree of identity (>80%) among

Table 1 Fungal *FKS* genes

Organism	GenBank Accession #	Reference	% identity to *S. cerevisiae FKS1*
Saccharomyces cerevisiae	U12893	[57]	—
Saccharomyces cerevisiae (FKS2)	U16783	[63]	87 %
Candida albicans	D88815	[29]	73 %
Candida glabrata	AF229171	Unpublished	83 %
Aspergillus nidulans	U51272	[6]	63 %
Aspergillus fumigatus	U79728	[27]	63 %
Cryptococcus neoformans	AF102882	[11]	56 %
Neurospora crassa (partial)	AF056047	Unpublished	99 %
Schizosaccharomyces pombe	D78352	[101]	52 %
Yarrowia lipolytica	AF198090	Unpublished	67 %
Coccidioides immitis (partial)	AF159533	Unpublished	65 %
Paracoccidioides brasiliensus	AF148715	[103]	63 %
Pneumocystis carinii	AF291999	[102]	61 %

Fungal *FKS* genes, the sequence of which (complete or partial) is in the GenBank database, are included. Percentage identity between each Fks protein and *S. cerevisiae* Fks1p was determined using the BlastN sequence comparison algorithm.

most of the known Fks protein sequences, and it has been proposed that this region must have some essential, conserved function [27,6]. All algorithms that have been used to map the topology of Fks proteins predict that this domain is on the cytoplasmic face of the plasma membrane. Interestingly, in the case of organisms whose genomic copy of *FKS* contains introns (*A. nidulans*, 2 [6]; *A fumigatus*, 2 [27]; *C. neoformans*, 7 [11]), there are no introns within this coding region; perhaps this portion of the sequence is derived from a common ancestral origin. Aside from the genetic analysis in *S. cerevisiae*, the suspected function and high degree of conservation among *FKS* genes suggest an essential role for cell viability, and direct attempts to disrupt single-locus *FKS* genes in *C. albicans*, *C. neoformans* and *A. fumigatus* have been reported [11,27,68]. In the diploid *C. albicans*, a two-step integrative disruption strategy failed to inactivate both copies of the gene—the plasmid designed to interrupt the *CaFKS1* coding sequence only inserted into the preexisting disrupted allele during a second round of integrative transformation, leaving a functional allele of the gene unperturbed. That the *CaFKS* gene product represents the target of the echinocandins was also established in this study [68]. Disruptions targeted to the *CaFKS1* locus of rare, heterozygous drug-resistant *C. albicans* mutants (isolated in a prior selection for resistance to L-733560 on agar plates) produced clones that were either fully resistant (because the wild-type allele of the locus had been disrupted) or fully susceptible (because the mutant allele of the locus had been disrupted). In *C. neoformans*, homologous integrative transformation at the wild-type *CnFKS1* locus was performed with a plasmid that could integrate in two orientations, only one of which would result in loss of gene function. None of the 23 homologous recombinants recovered in this screen had a disrupted copy of *CnFKS1* [11]. Finally, a strategy that has been used successfully for disruption of more than 10 nonessential genes in *A. fumigatus* (with 5–10% efficiency) yielded no *AfFKS1* knock outs among 105 transformants screened [27]. With molecular genetic analysis providing evidence that an Fks protein is indispensable in organisms from four different fungal genera, efforts to pinpoint its function are likely to continue.

There are several lines of evidence implicating Fks1p and Fks2p as subunits of β(1,3)-glucan synthase. First, the predicted membrane localization of both proteins is consistent with biochemical characterization of the enzyme. A subunit of the complex from *H. anomala* that was not extracted from crude membranes with salt and detergent was protected from heat inactivation by adding UDP-glucose, indicating that the catalytic centre resides in an integral membrane protein [17]. For two independent mutants resistant to GS inhibitors [*S. cerevisiae* R560-1C (*etg1-1*) and *S. pombe* JCR18 (*pbr1-8*)], fractionation and reconstitution experiments revealed that resistance is associated with an integral membrane protein [18,20]. Second, the Fks1 protein from *S. cerevisiae* [28], *A. nidulans* [6], *C. albicans* [29] and *A. fumigatus* [27] co-purifies with enzyme activity during solubilization and product entrapment, and there are no reports of samples with β(1,3)-glucan synthesis activity that lack a member of the Fksp family. Third, disruption of the *FKS1* gene from *S. cerevisiae* produces mutants that grow slowly [57], with morphological changes consistent with cell wall defects [59] and a reduction in β(1,3)-glucan synthase activity [57]. Fourth, immunoprecipitation of the Fks1 protein from *S. cerevi-*

siae enzyme preparations causes depletion of solubilized enzymatic activity from supernatants, and activity is recovered in pellets [69]. Fifth, immunofluorescent staining of the Fks1 protein from either *S. cerevisiae* or *A. fumigatus* revealed a concentration of protein at the site of active β(1,3)-glucan synthesis; for *S. cerevisiae*, this is the bud tip during polarized growth and the bud neck during cytokinesis [70,71], and for *A. fumigatus* this is the hyphal apex [27]. Lastly, direct interaction between Fks proteins and Rho1p has been reported [72]. Rho1p itself associates with protein kinase C (Pkc1p), an important regulatory protein that is responsible for maintenance of cell-wall integrity through activation of the mitogen-activated protein kinase (MAPK) cascade. These interactions were discovered through either co-immunoprecipitation experiments [7,9] or use of the yeast two-hybrid system [73].

If members of the Fks family of proteins provide the catalytic centre of β(1,3)-glucan synthase, they represent a significant divergence from known glycosyl transferases that use a nucleotide-diphospho (NDP)-sugar as substrate. A collection of over 550 protein sequences defined as NDP-sugar hexosyltransferasaes was subjected to hydrophobic cluster analysis, a sequence comparison method that can group functionally-related proteins displaying limited sequence relatedness into families [74]. Although many proteins from *S. cerevisiae*, including mannosyltransferases and chitin, glycogen and trehalose-phosphate synthases were identified and categorized in this analysis, the Fks proteins were conspicuously absent. Neither of two proposed UDP-glucose binding sites ((R/K)XGG implicated from glycogen synthases [75] or D,D,D35QXXRW from hydrophobic cluster analysis [76,77]) is found in Fks1p. In contrast, a processive β(1,3)-glucan synthase from an *Agrobacterium* strain was cloned and sequenced , and it does contain the latter motif [78]. The gaps in our knowledge of β(1,3)-glucan synthase mechanism provoke many questions (does polymerization proceed from the non-reducing end of the growing chain? Are glucose residues added as monomers or as disaccharide units?). Uncertainties about the structure-function relationships of glycosyltransferases in general make it difficult to confirm or refute a direct role for Fks proteins in catalysis. To date, only three crystal structures of glycosyltransferases have been solved [79,80,81] but a consensus nucleotide-binding domain, consisting of an α/β/α-layer fold, has been proposed [82]. Aspartate or glutamate residues within this pocket could be contact residues for the bound NP-sugar. Along these lines, Mouyna et al. [83] used hydrophobic cluster analysis and site-directed mutagenesis to confirm two glutamate residues as essential for catalytic activity in a glycosyl phosphatidylinositol-anchored β(1,3)-glucanosyltransferase from *A. fumigatus*. Similar analysis of predicted domains of the Fks proteins may provide insight into their function.

Characterization of spontaneous mutations in *FKS* genes from *S. pombe*, *S. cerevisiae* and *C. albicans* has led to identification of two residues within Fks proteins that are critical for function, and others that are necessary for susceptibility to inhibitors of glucan synthesis. The *S. pombe* strain MBY159 contains a temperature-sensitive mutation in *FKS1* (*drc1-191*) that blocks septum formation—cells die as a result of failed cytokinesis at 36C [84]. The mutation causes asparagine to be substituted for a conserved aspartate (D392 of *S. cerevisiae* Fks1p). Another loss-of-function mutation, the *fks1-1* allele of *S. cerevisiae*, results from a D441K replacement, and this change seems to confer identical phenotypes to those seen with insertion–deletion null alleles (C.M. Douglas, S.A. Parent, W. Li, G. Chrebet, J.A. Marrinan and M.B. Kurtz, unpublished results). Among fungal isolates selected *in vitro* for resistance to β(1,3)-glucan synthesis inhibitors, four independent *S. cerevisiae* echinocandin-resistance point mutations [*fks1-2* (*etg1-1*) from strain R560-1C, *fks1-3* (*etg1-3*) from strain MS10, *fks1-4* (*etg1-4*) from strain MS14, and *fks2-1* from strain YFK978] have been localized (C.M. Douglas, S.A. Parent, W. Li, G. Chrebet, J.A. Marrinan and M.B. Kurtz, unpublished results). Three of these four *S. cerevisiae* resistance mutations define an eight amino-acid cluster within the consensus Fksp sequence (Table 2), and several of these residues are conserved among all Fks proteins [11]. The fourth echinocandin-resistance mutation, *fks1-4*, maps outside of the cluster (position 1357) and is unique phenotypically because of the concomitant Nikkomycin Z hypersensitivity it confers [34,61]. Site-directed mutagenesis has confirmed that the substitutions identified by DNA sequencing are responsible for the resistance phenotypes. Work with the *C. albicans* L-733560-resistant mutants CAI4-R1, NR2, NR3 and NR4 [68] focused on the region of *CaFKS1* encoding amino acids 641–648 of CaFks1p (Table 2). The sequence analysis uncovered similar but non-identical changes in this region (R. Kelly, E. Register, V. Vyas and M.B. Kurtz, unpublished results). Molecular analysis of these *CaFKS1* alleles confirm earlier findings that strains CAI4-R1, NR2 and NR4 are heterozygous, whereas strain NR3 seems to be a rare homozygous mutant; only strain NR3 exhibited significant resistance to L-733,560 in an animal model of disseminated candidiasis [68,85]. From researchers at Eli Lilly & Co., a parallel selection for *S. cerevisiae* mutants resistant to the echinocandin B-derivative LY280949 also identified substitutions in the *FKS1* gene [86], and the authors suggest that these

Table 2 Fks protein alignment in a region defined by echinocandin-resistance mutations

ScFks1p:	Phe_{639}	Leu	Val	Leu	Ser	Leu	Arg	Asp_{646}
ScFks1-2p:	**Ile_{639}**	Leu	Val	Leu	Ser	Leu	Arg	Asp_{646}
ScFks1-3p:	Phe_{639}	Leu	Val	Leu	Ser	Leu	Arg	**Tyr_{646}**
ScFks2p:	Phe_{658}	Leu	Ile	Leu	Ser	Leu	Arg	Asp_{665}
ScFks2-1p:	Phe_{658}	Leu	**Lys**	Leu	Ser	Leu	Arg	Asp_{665}
CaFks1p:	Phe_{641}	Leu	Thr	Leu	Ser	Leu	Arg	Asp_{648}
CgFks1p:	Phe_{655}	Leu	Ile	Leu	Ser	Leu	Arg	Asp_{662}
AnFks1p:	Phe_{671}	Leu	Thr	Leu	Ser	Ile	Lys	Asp_{678}
AfFks1p:	Phe_{674}	Leu	Thr	Leu	Ser	Phe	Lys	Asp_{681}
CnFks1p:	Phe_{533}	Leu	Thr	Leu	Ser	Phe	Arg	Asp_{540}
SpFks1p:	Phe_{523}	Leu	Thr	Leu	Asn	Leu	Ala	Asp_{530}
YlFks1p:	Phe_{692}	Leu	Ile	Leu	Ser	Leu	Arg	Asp_{699}
CiFks1p:	Phe_{641}	Leu	Thr	Leu	Ser	Ile	Lys	Asp_{648}
PbFks1p:	Phe_{670}	Leu	Thr	Leu	Ser	Phe	Arg	Asp_{677}
PcFks1p:	Phe_{711}	Leu	Ser	Leu	Ser	Leu	Arg	Asp_{718}

Predicted Fksp sequences were aligned as described in Table 1. Abbreviations of protein names refer to the genus and species of each organism, with the same order (descending) as shown in Table 1; *N. crassa* Fks1p is omitted owing to incomplete sequence data. Mutant Fks protein sequences from the S. cerevisiae *fks1-2* (*etg1-1*), *fks1-3* (*etg1-3*) and *fks2-1* alleles described in the text are listed as ScFks1-2p, ScFks1-3p and ScFks2-1p, respectively. Specific amino-acid substitutions in these proteins are in bold. Subscripts refer to residue positions in primary amino-acid sequences. The accession numbers are as in Table 1, except for CaFks1p (Genbank # AF027295).

mutations lie within an ''echinocandin binding domain'' encompassing amino-acids 583–672 of Fks1p [87]. However, there are no reports in the literature describing direct interaction between any inhibitor of β(1,3)-glucan synthesis and an Fks protein.

Evaluation of product-entrapped and solubilized enzyme from resistant mutants may provide insight into the mechanism of echinocandin inhibition. Researchers at Merck & Co. (J. Nielsen Kahn and M-J. Hsu, personal communication) have been exploring the kinetics of synthesis, and inhibition, with enzyme derived from *S. cerevisiae* wild-type and mutant strains. Several advantages of using purified enzyme are immediately apparent; the IC_{50} for caspofungin inhibition is more in keeping with its minimum inhibitory concentration against whole cells, the enzyme inhibition curve approaches 100% inhibition at the highest drug levels, and the K_M for substrate UDP-glucose is lower than the value of 1 mM seen when crude membranes are used. With radiolabelled caspofungin as a tool, competition between classes of inhibitors (lipopeptides, papulacandins and sterol glycosides) has been assessed, and the relationship between drug resistance and inhibitor dissociation constant is being explored. Some structure-function relationships for Fks proteins are likely to be resolved through these studies.

Regulation of β(1,3)-glucan synthesis

As a process intimately associated with cell growth and morphogenesis, it is not surprising that synthesis of cell-wall polysaccharides is regulated tightly. To control β(1,3)-glucan synthase, cells can modulate catalytic activity either directly, by controlling the quantity or membrane localization of GTP-bound (activated) Rho1p in the enzyme complex, or indirectly through activation or repression of transcription. Signal transduction pathways that impinge upon cell-wall biosynthesis are discussed in detail in the review by Pla et al of this issue. A brief overview of regulatory mechanisms that are involved specifically in controlling β(1,3)-glucan synthase is presented here.

S. cerevisiae β(1,3)-glucan synthesis activity requires Rho1p, a protein that interacts not only with both Fks proteins but also with protein kinase C (Pkc1p), which is a known regulator of the MAPK cascade and actin-cytoskeleton assembly pathway in yeast [69,71,72,73,88]. Because the Pkc1 protein, like Rho1p and Fksp, is found in highly purified preparations of the enzyme, it could be considered a subunit of the complex [72]. As a multifunctional regulator, Rho1p acts as a 'switch' that must interface with numerous proteins, including guanine-nucleotide exchange factors encoded by *ROM1* and *ROM2* [70] that can exchange Rho1p-associated GTP for GDP, and GTPase-activating proteins such as Bem2p, Sac7p and Lrg1p, which stimulate a transition from GTP-bound active Rho1p to the less active GDP-bound form [89]. Rho1p also must be modified post-translationally through prenylation to stimulate β(1,3)-glucan synthesis activity, and to bind directly to Fks1p [90]. Presumably, membrane localization of Rho1p requires prenylation, which is catalyzed by the enzyme

geranylgeranyl transferase I [91]. The role for Rho1p in regulating β(1,3)-glucan synthase has been most thoroughly studied in *S. cerevisiae*, but homologues of *RHO1* from *S. pombe* [92] and pathogenic fungi such as *C. albicans* [21], *A. fumigatus* [27] and *C. neoformans* [93] have also been described. Overall, the paradigms established in *S. cerevisiae* are echoed in other fungi, with some notable differences in *C. neoformans*. Even though the *CnRHO1* homologue is an essential gene in *C. neoformans*, neither overexpression nor replacement of the resident copy with a temperature sensitive allele of *CnRHO1* led to the same phenotypes in *C. neoformans* as were observed when similar studies were performed with *S. cerevisiae* or *S. pombe* [94]. These differences indicate that Rho1p may have a somewhat different role in regulating β(1,3)-glucan synthase activity in *C. neoformans*. The nature of the interaction between Rho1 proteins and Fks proteins is still largely undefined for all organisms.

Transcriptional regulation of *FKS* gene expression in *S. cerevisiae* reflects a complex circuitry that must respond to very diverse signals of stress, growth and development. During vegetative growth, *FKS1* is preferentially expressed over *FKS2*, and *FKS1* mRNA levels fluctuate periodically during the cell cycle [62]. Promoter elements upstream of the *FKS1* open reading frame, including an MCB (*Mlu*I cell-cycle box) and several cell-cycle boxes sequences, match elements associated with cell-cycle regulation of many genes. It is because *FKS1* expression predominates when cells are grown logarithmically on rich medium that echinocandin resistance mutations selected under these conditions map to *FKS1*; the only example of an *FKS2* mutation (*fks2-1*) conferring whole-cell and enzyme resistance to L-733560 was selected in a strain without a functional copy of *FKS1* (C.M. Douglas, S.A. Parent, W. Li, G. Chrebet, J.A. Marrinan and M.B. Kurtz, unpublished results). Characterization of *FKS2* expression shows clearly that Fks2p is required when cells are stressed by high temperature, growth in glucose-depleted medium loss of Fks1p or when cells respond to mating factor or initiate sporulation [63,95]. Dissection of the transcriptional machinery required for these responses is ongoing but some elegant models, integrating known and novel transcription factors and response elements, have developed. First, components of the cell-integrity pathway were identified in a screen for suppressors of the synthetic lethality of a mutant lacking both calcineurin B and *FKS1*. These genes (*MKK1*, a mitogen-activated protein kinase kinase homologue; and *RLM1*, responsible for resistance to lethality of $MKK1^{P386}$) can induce *FKS2* expression when overexpressed in the *cnb1*Δ *fks1*Δ double mutant, and both *MKK1* and *RLM1* function in a cell-integrity pathway mediated by *RHO1* and *PKC1*. Rho1p therefore must have a dual role as both a subunit of the β(1,3)-glucan synthase enzyme complex and as a regulatory component affecting *FKS2* gene expression [95]. Second, *FKS2* is upregulated when wild-type *S. cerevisiae* is grown in glucose-deficient medium. This response is mediated through the *SNF1*-encoded kinase (required for derepression of many glucose-repressible genes) and *MIG1*, a transcriptional repressor that is a target of *SNF1*. *FKS2* thus joins a family of genes that are controlled through a well characterized pathway capable of responding to changes in extracellular glucose [95]. Promoter mapping experiments have begun to discriminate specific sequences that are required for both of these responses [96].

Themes relating calcineurin regulation to β(1,3)-glucan synthesis have been reiterated in work with *C. neoformans* [97,98]. This organism exhibits poor *in vitro* susceptibility to all inhibitors of GS β(1,3)-glucan synthase, including echinocandins, papulacandins and sterol glycosides, despite evidence that the *CnFKS1* gene is both single-copy and essential [11]. A dramatic enhancement of echinocandin *in vitro* activity against *C. neoformans* was observed when the compound was used in combination with calcineurin inhibitors such as FK506 or L-685818, a non-immunosuppressant analogue. This phenotype is mediated through an FK506-binding protein known as FKBP12. Disruption of genes encoding the FK506 target proteins calcineurin and FKBP12 produced a strain that is hypersensitive to caspofungin, further supporting the notion that calcineurin may have a role in *CnFKS1* biology. Although elimination of calcineurin function does confer temperature-, CO_2- and pH-sensitive growth *in vitro*, and loss of virulence in an animal model of cryptococcal meningitis, it is not lethal when cells are grown in culture at 30°C [97]. If *CNFKS1* expression is regulated directly by calcineurin, as with *FKS2* from *S. cerevisiae*, there must be sufficient CnFks1p synthesis to support growth in strains lacking calcineurin. Concurrent work with *C. albicans* has illustrated a role for calcineurin in susceptibility to the azole antifungal fluconazole [99]; future studies may define combinations of antifungal agents, including echinocandins, azoles and non-immunosuppressant analogues of FK506, which provide a broader spectrum of antifungal activity than that seen with these agents alone.

Conclusions

The enzyme β(1,3)-glucan synthase is a validated target for human antifungal therapy, a well-conserved component of fungal morphogenetic machinery, and a downstream effector of complex networks that regulate

responses to both growth signals and stress. The expanding horizon of research in this area includes a recent description of a cell plate-specific callose synthase from *Arabidopsis* (CalS) that exhibits strong homology to the Fks protein family [100], and identification of intermediates in the *S. cerevisiae* sphingolipid pathway, which act as non-competitive inhibitors (regulators?) of enzyme activity [62]. With many questions about the biology of this enzyme still unresolved, it is likely that strong interest in β(1,3)-glucan synthesis will remain.

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