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Isolation of a Gene Involved in 1,3-β-Glucan Synthesis in *Aspergillus nidulans* and Purification of the Corresponding Protein

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***Saccharomyces cerevisiae* has two highly homologous genes, *FKS1* and *FKS2*, which encode interchangeable putative catalytic subunits of 1,3-β-glucan synthase (GS), an enzyme that synthesizes an essential polymer of the fungal cell wall. To determine if GS in *Aspergillus* species is similar, an *FKS* homolog, *fksA*, was cloned from *Aspergillus nidulans* by cross-hybridization, and the corresponding protein was purified. Sequence analysis revealed a 5,716-nucleotide coding region interrupted by two 56-bp introns. The *fksA* gene encodes a predicted peptide of 229 kDa, FksAp, that shows a remarkable degree of conservation in size, charge, amino acid identity, and predicted membrane topology with the *S. cerevisiae FKS* proteins (Fksps). FksAp exhibits 64 and 65% identity to Fks1p and Fks2p, respectively, and 79% similarity. Hydropathy analysis of FksAp suggests an integral membrane protein with 16 transmembrane helices that coincide with the transmembrane helices of the *Saccharomyces* Fksps. The sizes of the nontransmembrane domains are strikingly similar to those of Fks1p. The region of FksAp most homologous to the *Saccharomyces FKS* polypeptides is a large hydrophilic domain of 578 amino acids that is predicted to be cytoplasmic. This domain is 86% identical to the corresponding region of Fks1p and is a good candidate for the location of the catalytic site. Antibodies raised against a peptide derived from the FksAp sequence recognize a protein of ~200 kDa in crude membranes and detergent-solubilized active extracts. This protein is enriched ~300-fold in GS purified by product entrapment. Purified anti-FksAp immunoglobulin G immunodepletes nearly all of the GS activity in crude or purified extracts when *Staphylococcus aureus* cells are used to precipitate the antibodies, although it does not inhibit enzymatic activity when added to extracts. The purified GS is inhibited by echinocandins with a sensitivity equal to that displayed by whole cells. Thus, the product of *fksA* is important for the activity of highly purified preparations of GS, either as the catalytic subunit itself or as an associated copurifying subunit that mediates susceptibility of enzymatic activity to echinocandin inhibition.**

Pneumocandins comprise a family of acylated cyclic hexapeptides which are active in vivo against *Pneumocystis*, *Candida*, and *Aspergillus* species (1, 36, 53, 54) and are promising candidates for treatment of life-threatening fungal infections. Their primary mode of action is inhibition of 1,3-β-glucan synthesis, as shown by both enzyme assay of cell extracts and whole cell effects. The multisubunit enzyme 1,3-β-glucan synthase (UDP-glucose:1,3-β-D-glucan 3-β-D-glucosyltransferase; EC 2.4.1.34; GS) catalyzes the formation of 1,3-β-D-glucan, a critical cell wall polymer, from UDP-glucose (UDPG) (15). In the human pathogen *Aspergillus fumigatus*, pneumocandins cause distension and swelling of the hyphae and a substantial change in their branching pattern (38). Inhibition of GS from *A. fumigatus* by several pneumocandins and by a related lipopeptide, cilofungin, has been demonstrated in vitro (7, 37, 38), and it is presumed that the morphological changes result from inhibition of 1,3-β-glucan synthesis in treated cells. We are interested in evaluating the echinocandin target and chose to study *Aspergillus nidulans* as a model for the closely related pathogenic fungus *A. fumigatus*. *A. nidulans* has the advantages of being a well-characterized nonpathogen amenable to genetic analysis. The recent demonstration that numerous genes from *A. fumigatus* can complement *A. nidulans* mutations supports the use of the latter organism as a model for *A. fumigatus* (11).

Two groups have reported extraction of crude GS from *A. fumigatus* by different detergents but have not reported further purification (7, 8). The only filamentous fungus from which highly purified GS has been obtained is *Neurospora crassa* (3, 4). The key to the purification was the use of product entrapment, a powerful technique for purifying enzymes synthesizing polymeric products that has also been used to isolate GS with similar purity from *Saccharomyces cerevisiae* (31).

The genetics of GS have been most extensively studied in *S. cerevisiae*, which is often used as a model for the asexual pathogen *Candida albicans*. Mutants of *S. cerevisiae* resistant to the pneumocandin L-733,560 have been isolated (19, 22). Evidence suggests that the mutation in the resistant mutant R560-1C (*etg1-1*) affects a subunit of β-glucan synthase which resides in the membrane-bound fraction, as originally defined by Kang and Cabib (33). Kang and Cabib demonstrated that GS from several fungi, both yeast and filamentous, could be fractionated into a particulate membrane-bound (catalytic) component (fraction B) and a soluble (GTP-binding) component (fraction A). A large gene that restored sensitivity to the R560-1C mutant, *ETG1* (for echinocandin target gene), is identical to *FKS1* (18). *FKS1* was isolated by complementation of an *S. cerevisiae* mutant hypersensitive to FK506 (18, 23) and has been independently cloned by several groups (16, 27, 31,

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47). Strains containing a disruption of *FKS1* are viable, have five- to eightfold-less GS activity in vitro, and are threefold more sensitive to L-733,560 (18). A second echinocandin target gene from *S. cerevisiae*, *FKS2*, was cloned by homology to *FKS1* and encodes a polypeptide 88% identical to Fks1p (42). Both Fks1p and Fks2p are integral membrane proteins. Disruption of *FKS2* alone is not lethal, but simultaneous disruption of both *FKS1* and *FKS2* is, suggesting that they encode interchangeable subunits of GS with common functions. Fks1p is the major protein responsible for GS activity during vegetative growth, while Fks2p appears to be important for sporulation (42).

Here we report the isolation of a homolog of *FKS1* and *FKS2* from *A. nidulans*, *fksA*, and greater than 300-fold enrichment of the corresponding protein (FksAp) with a concomitant increase in GS specific activity. We further demonstrate that FksAp is involved in glucan synthesis and is inhibited by pneumocandins. By providing genetic and biochemical evidence of the target of pneumocandins in an *Aspergillus* species, the results of this study afford further support for the use of this class of antifungal agents in clinically important aspergillosis.

MATERIALS AND METHODS

Polyoxyethylene ether W1, Tergitol Nonidet P-40 (NP-40), laminarinase (*Penicillium* sp.), and laminaroligosaccharides (mixed anomers up to seven) were obtained from Sigma.

Strains, media, and microbiological methods. *A. nidulans* cosmid libraries constructed in vectors pWE15 and pLORIST2 (14) and FGSC 4, a wild-type strain of *A. nidulans* (Glasgow wild type), were obtained from the Fungal Genetics Stock Center, Kansas City, Kans. MF5668 is a clinical isolate of *A. fumigatus*. Plasmids used in this study were constructed by standard recombinant DNA techniques (50). Standard medium components were obtained from Difco. YNBD medium is yeast nitrogen base with 2.0% glucose. GYEP medium contains 0.3% yeast extract, 1.0% peptone, and 2.0% glucose. YME contains 0.4% yeast extract, 1.0% malt extract, and 0.4% dextrose. For determination of the in vitro susceptibility of aspergilli to pneumocandins, 10^4 conidia were inoculated into 0.15 ml of YNBD medium containing twofold serial dilutions of the test compounds as described previously (38). The morphological changes were determined visually after incubation at 37°C for 48 h. For isolation of genomic DNA and total RNA from *A. nidulans*, 5×10^8 conidia were inoculated into 500 ml of GYEP medium and incubated at 37°C for 16 h with shaking at 200 rpm. For enzyme extraction, conidia were harvested from the surface of potato dextrose agar after 5 to 7 days of growth at 37°C, suspended in 0.01% Tween 20, and enumerated. Liquid cultivation was performed by inoculating 2×10^6 conidia per ml in YME and shaking them rapidly at 37°C for 14 to 16 h. *S. cerevisiae fks1* and *fks2* null mutants YFF2421 and YFF2714 were grown as described previously (18, 42).

Nucleic acid isolations and hybridizations. Genomic DNA was isolated by a protocol described by Tang et al. (57), with the inclusion of a lyophilization step prior to breakage of the mycelia. All DNA blots were performed with Zeta-ProbeGT derivatized nylon membranes (Bio-Rad) and were hybridized under stringent conditions recommended by the manufacturer. Total RNA was isolated as described by Timberlake (58) and separated on 1.0% formaldehyde-agarose gels (50). RNA was transferred to Nytran Plus membranes (Schleicher & Schuell), and hybridization was performed at 65°C as described previously for DNA slot blots (34). Probes were radiolabeled with [α-^{32}P]dCTP, using a random-primed DNA labeling kit (Stratagene).

Nucleotide sequence analysis. The DNA sequence of both strands of ~6.7 kb of *fksA* was determined with Sequenase version 2.0 (United States Biochemical) or on a model 373A automated DNA sequencer with a Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). The *fksA* gene was localized by transcript mapping, and this information was used to design a sequencing strategy. Sequencing of the cloned 568-bp *Pst*I-*Eco*RV fragment was initiated in both directions, using primers complementary to the vector. The sequence was extended with *fksA*-derived primers by using various plasmid DNAs as templates, and the remainder of the sequence was obtained by sequencing plasmids containing nested deletions that were constructed as described by Gewain et al. (29). The sequence was analyzed with the Genetics Computer Group software package (28). Percent identity between protein sequences was determined with the GAP program.

An intron search was conducted by scanning the entire sequence for the 5′ and 3′ internal intron consensus sequences GTANGT (6) and CTRAC (21, 40), respectively, and then for specific 5′ splice sites and 3′ internal intron sequences from numerous *A. nidulans* introns. Regions where the peptide homology to the *S. cerevisiae* Fksps was poor were further scrutinized for potential splice sites.

RNase protection assay. RNase protection assays were performed by using an RNase protection kit from Ambion. A 719-bp antisense RNA probe (corresponding to nucleotides 547 to 1246 plus an additional 20 bp from vector) encompassing the putative intron that is located from nucleotides 829 to 885 was synthesized and hybridized overnight to 30 μg of total RNA from *A. nidulans*. Hybrids were analyzed as instructed by the manufacturer.

Assay and initial extraction of GS. GS activity was determined by a modification of the method of Kang and Cabib (33). The final assay mixture (100 μl) contained 50 mM Tris (pH 7.5), 10% glycerol, 1.5 mg of bovine serum albumin per ml, 25 mM KF, 1 mM EDTA, 10 μM GTPγS, 1 mM EDTA, 1 U of α-amylase, 2 mM UDP-[^{3}H]glucose (2,000 dpm/nmol), and enzyme (20 to 50 μg of crude membranes, 5 to 10 μg of detergent-extracted GS, or 0.1 to 0.5 μg of purified GS). Reactions were started by addition of substrate, incubated at 22°C for 30 to 90 min with gentle rocking, and stopped by the addition of 100 μl of 20% trichloroacetic acid. Acid-insoluble product was collected on 96-well glass-fiber filters. Radioactivity was determined by liquid scintillation counting with 40% efficiency, using 10 ml of Optiphase HiSafe 3 scintillant (Wallac). Protein concentrations were determined by the method of Bradford (13) with Bio-Rad reagent or by the bicinchoninic acid method reagent (Pierce). Bovine serum albumin (Pierce) was used as standard for both methods.

Mycelia were harvested by filtration over Miracloth (Calbiochem) and washed extensively with water. For some purposes, washed mycelia were stored at −80°C for later breakage, but the highest specific activities of GS were obtained by immediate disruption of fresh cells cultured for 14 to 16 h.

Cell breakage was performed by two passages through a French press at 12,000 lb/in^2 in chilled extraction buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 1 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml, and 10 μM GTPγS (extraction buffer) at approximately 3 ml of buffer per g (wet weight). After centrifugation at 2,500 × *g* for 10 min to remove cell debris, membranes were sedimented at 100,000 × *g* for 1 h, washed once in extraction buffer by resuspension in a close-fitting Dounce homogenizer, and resedimented at 100,000 × *g* for 1 h. For purification, crude membranes were resuspended at 5 mg of protein per ml in storage buffer (50 mM HEPES [pH 7.5], 1 mM EDTA, 1 mM DTT, 20% glycerol) containing 0.025% Tergitol NP-40, mixed gently by inversion for 15 min at room temperature, and sedimented at 150,000 × *g* for 1 h. This step does not solubilize GS activity but partially depletes the membranes of the GTP-associated activating factor A and removes loosely associated proteins. Solubilization of activity was accomplished by resuspension of the depleted pellet in 50 mM HEPES (pH 7.5)–1 mM EDTA–1 mM DTT–20% glycerol at 5 mg of membrane protein per ml and adjustment to 0.3% (wt/vol) polyoxyethylene ether W1. After 30 min of gentle mixing at room temperature, insoluble material was sedimented at 150,000 × *g* for 1 h, and the clear supernatant was removed. This extract is referred to as depleted W1 in Table 1.

Purification of GS by product entrapment. Product entrapment was performed immediately after solubilization by adding NP-40 to a final concentration of 1.5%, KF to a final concentration of 25 mM, and UDPG to 2 mM. After 30 min of incubation at room temperature, glucan was sedimented at 3,000 × *g* for 10 min. The copious loosely packed pellet was resuspended in extraction buffer containing 0.5 mM UDPG, washed three times (3,000 × *g*, 10 min) in the same buffer, and then washed twice in buffer without UDPG. The final pellet was harvested at 15,000 × *g* after transfer to a microcentrifuge tube. Solubilization after entrapment was performed by resuspension of the pellet in storage buffer containing 0.1% W1 and centrifugation at 60,000 × *g* for 15 min. The supernatants of three such washes were combined and referred to as the soluble product-entrapped (PE) enzyme. The final pellet was resuspended in a minimal volume of storage buffer containing 0.05% NP-40 and stored at −70°C.

Reconstitution of enzyme from soluble components. Enzymatic activity was reconstituted from fully solubilized separated fractions. Crude particulate membranes, isolated as described above but without added GTPγS, were resuspended at 10 mg/ml in extraction buffer containing 0.025% NP-40 (wt/vol), incubated at 4°C with intermittent mixing on a vortex shaker for 5 min, and centrifuged at 170,000 × *g* for 1 h. The supernatant was termed fraction A. For extraction of the bound membrane activity, fraction B, the pellet was resuspended at 10 mg of protein per ml in extraction buffer containing 0.8% W1, mixed at room temperature for 30 min, and centrifuged at 100,000 × *g* for 1 h. Gel filtration of the resulting supernatant on Sephadex G-75 in 0.05 M KPO_4 buffer (pH 7.5) containing 0.02% NP-40 removed residual A fraction.

Antibody preparation. A 14-residue peptide (EELTTDNVSPYTPG, amino acids 1154 to 1167) was chosen from the deduced sequence of FksAp by an antigenicity index search performed as described by Jameson and Wolf (32). It corresponds approximately to the region of *Saccharomyces* Fks1p and Fks2p against which antibodies were successful in recognizing the yeast proteins. Polyclonal antibodies were generated to a conjugate with bovine thyroglobulin as described previously (42) in four rabbits (HRP, Inc., Denver, Pa.). Western blotting (immunoblotting) was performed with a 1:5,000 dilution of serum and 10 μg of *Aspergillus* W1 extract.

Immunoprecipitation. Antiserum was affinity purified by passage over the *Aspergillus*-specific peptide immobilized on sulfo-MBS (sulfo-*m*-maleimobenzoyl-*N*-hydroxysuccinimide ester) resin (Pierce) as previously described (42). GS in immunoprecipitation buffer (phosphate-buffered saline [PBS] containing 1% NP-40) was incubated with antiserum for 30 min at room temperature prior to

the addition of 25 μl of a 10% (wt/vol) suspension of attenuated *Staphylococcus aureus* cells (Pansorbin cells; Calbiochem). After 1 h of incubation at 4°C, the cells were removed by centrifugation and the supernatant was assayed for GS activity.

Nucleotide sequence accession number. The nucleotide sequence of *fksA* has been assigned GenBank accession number U51272.

RESULTS

***A. nidulans* as a model for studying the echinocandin target of *A. fumigatus*.** *A. nidulans* wild-type strain FGSC 4 was compared with *A. fumigatus* MF5668, a strain used for animal models of disseminated aspergillosis, for sensitivity to pneumocandins in vitro. The minimum effective concentrations that altered growth and morphology (38) were the same for both strains: 4.0 μg/ml (3,800 nM) with the natural product, L-688,786 (55), and 0.002 μg/ml (1.7 nM) with the semisynthetic analog L-733,560 (12). Identical morphological effects were also seen for both strains; i.e., the hyphae grew abnormally, producing compact clumps instead of the highly branched filaments that were observed at concentrations of inhibitor below the minimal effective concentration.

The inhibitory effects of L-688,786 and L-733,560 on in vitro GS activity in crude membranes of *A. nidulans* were quantitated. Fifty percent inhibitory concentrations (IC_{50}s) of 1,000 and 8 nM were determined for L-688,786 and L-733,560, respectively. The relative potency of these two pneumocandins with respect to inhibition of in vitro GS correlated well with the whole cell susceptibility results. All of these data indicate that studies of the echinocandin target of *A. nidulans* would be relevant to *A. fumigatus*.

Cloning a homolog of the *S. cerevisiae* echinocandin target gene. Preliminary Southern blot analysis with probes from *S. cerevisiae FKS1* (4.0-kb *Kpn*I-*Kpn*I fragment of pJAM54 [18]) and *FKS2* (1.7-kb *Pst*I-*Kpn*I fragment from pFF250 [42]) indicated that both hybridized to the same restriction fragments of genomic DNA from *A. nidulans*. A cosmid library of *A. nidulans* genomic DNA was screened with the *FKS2* probe, and one clone that hybridized strongly, pGS1, was isolated. Southern blot analysis indicated that pGS1 was not completely colinear with genomic DNA. A 568-bp *Pst*I-*Eco*RV fragment was determined to be colinear and internal to the hybridizing region. An additional cosmid library was screened with the homologous 568-bp *Pst*I-*Eco*RV fragment. Southern blot analysis of one clone, p11G12, indicated that sites for all of the restriction enzymes that we tested were colinear with genomic DNA and that a minimum of 15 kb of the cloned DNA was colinear (data not presented). The *A. nidulans* homolog has been designated *fksA*.

Expression and sequence analysis of *fksA*. To determine whether *fksA* was expressed, Northern (RNA) blot analysis was performed with the 568-bp *Pst*I-*Eco*RV fragment as the probe. A single transcript of 7.0 kb that is slightly larger than the corresponding *S. cerevisiae* transcripts was detected (Fig. 1) (18, 42).

The DNA sequence of *fksA* is shown in Fig. 2. Translation of the DNA sequence revealed two regions in different reading frames with substantial homology to the *S. cerevisiae FKS* proteins. Examination of the DNA sequence for possible intervening sequences revealed a 56-bp putative intron interrupting these two homologous regions at amino acid 1806 (Fig. 2). A second putative intron that interrupts a reading frame at amino acid 53 in the N terminus, a region with little similarity to the yeast proteins, was found.

Evidence for the putative intron at the 3′ end of the gene is as follows: (i) 5′ and 3′ internal splicing signal sequences of GTAAGA and CTAAC, respectively, which are in close agreement with the splicing consensus sequences, were found; (ii) a stop codon which interrupts an open reading frame with substantial homology to the *S. cerevisiae FKS* polypeptides is present at the beginning of the intron; and (iii) excision of the intron results in a frameshift which maintains good homology to the *FKS* proteins.

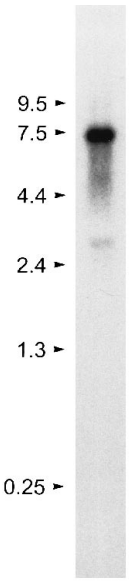

FIG. 1. Autoradiogram of Northern blot hybridization of total RNA from FGSC 4 to the radiolabeled 568-bp *Pst*I-*Eco*RV fragment of *fksA*. A 0.24- to 9.5-kb RNA ladder (GIBCO BRL) was included as a molecular size standard.

The putative intron at the 5′ end of the gene begins at position 829 and has 5′ and 3′ internal consensus sequences of GTAACT and CTGAT, respectively. There are two possible translational start sites in different reading frames (positions 671 and 1042). The one producing a peptide sequence with greater homology to the *S. cerevisiae FKS* polypeptides requires splicing of the putative intron. RNase protection assays were performed to determine whether the N-terminal intron sequence is absent in the mature mRNA. We expected to obtain a 361-bp protected fragment corresponding to the region 3′ of the intron and another protected fragment corresponding to the region 5′. Consistent with this, two protected fragments of approximately 360 and 170 bp were detected (data not presented). We have therefore concluded that the intron is absent in the mature mRNA and that translation most likely begins at nucleotide 671 (Fig. 2).

There is a third possible 57-bp intron spanning nucleotides 586 to 642 which could be entirely within the leader sequence of the gene. If this putative intron were spliced out, we would expect a 187-bp protected fragment from the region between the two 5′ introns to be produced. This is within the error of determination of the ~170-bp fragment. Alternatively, an ~170-bp fragment would be obtained if transcription started ~170 nucleotides upstream of the confirmed intron (this corresponds to ~11 nucleotides upstream of the putative translational start site). The position of the putative TATA boxes shown in Fig. 2 is compatible with transcription starting either just upstream of the proposed start codon or just upstream of the third possible intron 5′ of the coding sequence.

Taking into account the two introns that interrupt the coding sequence, we have proposed an open reading frame of 1,905 amino acids encoding a peptide with a molecular mass of 229 kDa. The sequence surrounding the putative translational

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   1  AGTCTGATTTTCCTGTTGGACAATCAGTTCCCTTTCCACTCTCGCTGACCGTCCAACTAACTTGTCTGCTCCCTCCCTTCGTCCTTCTCTTAGTTTCCTTTCTTTCTTTTCGAGGCTTCA
 121  CTTTTTCTTCAGTTGCTTTTGAGTCCCAGTCCGCTCTTTTTCATCGTTTGTACTCGGAAGCTTATCGACCACGGACCGTACTCCCCTTCCTTCCTTGTGCCTGTTGTTCGGGGAAAGAAG
 241  AGGAGGCGAGGCTGCGGTGCCCCTTCCTGTTGGGTCAAAAGAGTTTCCTCTCGTTATCTTCCATCATCATTCGTTTGACAATTTTTCAGAATCGATCGAGAACCTCTTCGAGTAGAAGAG
 361  GACAAAAGAATCCTTCACGAATCGTCGCTTTTCGATTGGGCTTGATAATACTACAAGGTTGACGGACAGGTCCGGTGCCTTCGAGCGATCGCCAACTCCGACTGTTTTCACGAAGCGCCT
 481  AGTGCATGATCTTTAGATTCGCTCGTCTATAACCGCTGATTTGTTCTGTCGATGCTATAATTACTGAAGCTTGATCGGACTAATATTTAATGGGCCTTTGTTTCTGTGAGTTTGGTTGTC
 601  TTGTTACTTGCATTTTTTGGGTTCAGAACTAACAGCAACAAGAGAACCTCGGTGATTGTCGCTTGTCATAATGTCGGGCTACCCACAAGGTTCGGGCTATCAGCACGGCAACTACGATGA
   1                                                                                                  M  S  G  Y  P  Q  G  S  G  Y  Q  H  G  N  Y  D  D

 721  TGGCTATGACCATCAGGGTCATGGCGATGCTTACTATCAAGATGACCACCATGCTCAAGCTGGTTATTATGATAACCAAGACTATGGGGATGGCTACTACGATAGAGGGTAACTTCCCTT
  18   G  Y  D  H  Q  G  H  G  D  A  Y  Y  Q  D  D  H  H  A  Q  A  G  Y  Y  D  N  Q  D  Y  G  D  G  Y  Y  D  R  G

 841  TATTCTAAAGACATGCCTCGCATTTGTGCTGATTTTTTTTTTAGTGGCTACGGGGATCATGGCTACCAGCATGATGGCTACTACGATGCGGGCCACCACGATGACTACTACGGCGATCAG
  54                                                 G  Y  G  D  H  G  Y  Q  H  D  G  Y  Y  D  A  G  H  H  D  D  Y  Y  G  D  Q

 961  TATTACGCCCAGGGAGGTCAAGGCAGAGGACGACATGGTCGTGACTCCGAGGAAGAGTCGGAGACCTTTAGTGATTTCACTATGAGGTCTGAAACAGCCCGTGCTGCCGACATGGACTAC
  79   Y  Y  A  Q  G  G  Q  G  R  G  R  H  G  R  D  S  E  E  E  S  E  T  F  S  D  F  T  M  R  S  E  T  A  R  A  A  D  M  D  Y

1081  TATGGGCGCGGGGACGAACGGTACAACAGTTACACTGATAGCCAGTACGGACACGGAGGCTACGGTTACCGCCCCCCTTCTTCGCAGGTGTCGTACGGTGGTAACCGGTCTTCTGGAGCG
 119   Y  G  R  G  D  E  R  Y  N  S  Y  T  D  S  Q  Y  G  H  G  G  Y  G  Y  R  P  P  S  S  Q  V  S  Y  G  G  N  R  S  S  G  A

1201  TCGACACCAGTGTATGGAATGGATTACGGCAATGGCTTGCCTGCAGGCCAGCGATCCCGAGAGCCATACCCTGCCTGGGCTTCCGATGGCCAAGTTCCGGTTTCAAAGGAGGAGATCGAA
 159   S  T  P  V  Y  G  M  D  Y  G  N  G  L  P  A  G  Q  R  S  R  E  P  Y  P  A  W  A  S  D  G  Q  V  P  V  S  K  E  E  I  E

1321  GACATTTTCATTGACCTTGTCAACAAATTCGGTTTTCAGCGGGACAGCATGCGCAACATGTACGACCATCTAATGACCCAGTTGGACTCCCGTGCCTCCCGCATGACTCCTAACCAGGCG
 199   D  I  F  I  D  L  V  N  K  F  G  F  Q  R  D  S  M  R  N  M  Y  D  H  L  M  T  Q  L  D  S  R  A  S  R  M  T  P  N  Q  A

1441  CTCCTCTCCCTTCATGCGGACTACATTGGTGGTGACAATGCCAACTACCGCCGTTGGTACTTCGCCGCCCATTTAGATCTCGACGATGCGGTTGGTTTTGCCAACATGAAACTGGGTAAA
 239   L  L  S  L  H  A  D  Y  I  G  G  D  N  A  N  Y  R  R  W  Y  F  A  A  H  L  D  L  D  D  A  V  G  F  A  N  M  K  L  G  K

1561  GCAGACCGCAAAACAAGAAAAGCTCGTAAGGCTGCGAAGAAGAAGGCACAAGAAAATCCAGAGGACGTCGAAGGAACGCTCGAGGCGCTGGAAGGCGACAATAGCCTAGAGGCCGCTGAA
 279   A  D  R  K  T  R  K  A  R  K  A  A  K  K  K  A  Q  E  N  P  E  D  V  E  G  T  L  E  A  L  E  G  D  N  S  L  E  A  A  E

1681  TACAGGTGGAAGACACGGATGAACCGTATGTCTCAGCATGAAAGGGTGCGCCAATTGGCACTGTACCTACTCTGTTGGGGCGAGGCCAACCAAGTCCGGTTTTTGCCCGAGGCTCTTTGC
 319   Y  R  W  K  T  R  M  N  R  M  S  Q  H  E  R  V  R  Q  L  A  L  Y  L  L  C  W  G  E  A  N  Q  V  R  F  L  P  E  A  L  C

1801  TTTATCTTCAAATGTGCCGACGACTTTTACAACTCGCCCGAGTGCCAGAACCGAGTGGAACCTGTTGAAGAGTTCACATATCTCAATGAGATCATCACTCCTCTGTATCAGTATTGCCGT
 359   F  I  F  K  C  A  D  D  F  Y  N  S  P  E  C  Q  N  R  V  E  P  V  E  E  F  T  Y  L  N  E  I  I  T  P  L  Y  Q  Y  C  R

1921  GACCAGGGTTATGAGATTTTGGATGGCAAGTATGTTCGCCGTGAGCGTGATCATAACCAGATCATTGGATATGATGACATGAATCAGCTCTTCTGGTACCCAGAGGGAATTGAGCGTATC
 399   D  Q  G  Y  E  I  L  D  G  K  Y  V  R  R  E  R  D  H  N  Q  I  I  G  Y  D  D  M  N  Q  L  F  W  Y  P  E  G  I  E  R  I

2041  GTGCTGGAGGACAAGACCCGCCTCGTTGACATTCCCACTGCAGAAAGGTGGACCAAGCTCAAGGAGGTGAACTGGAAGAAGGTCTTCTTCAAGACTTACAAGGAGACACGTTCCTGGTTC
 439   V  L  E  D  K  T  R  L  V  D  I  P  T  A  E  R  W  T  K  L  K  E  V  N  W  K  K  V  F  F  K  T  Y  K  E  T  R  S  W  F

2161  CATCTGGTTACAAACTTCAACCGTATCTGGGTCATCCATCTGGGTGCTTTCTGGTTTTTCACCGCGTATAACGCCCCTACCATTTACACTAAGAACTACAGACAGCAGCTCAACAACAAA
 479   H  L  V  T  N  F  N  R  I  W  V  I  H  L  G  A  F  W  F  F  T  A  Y  N  A  P  T  I  Y  T  K  N  Y  R  Q  Q  L  N  N  K

2281  CCACCAGGAGCTTACTACTGGTCTGCTGTTGGTTTTGGTGGAGCTTTGGTCTCATTCATCCAGATCATGGCGACTATCTTTGAATGGATGTATGTTCCTCGACGTTGGGCGGGTGCTCAG
 519   P  P  G  A  Y  Y  W  S  A  V  G  F  G  G  A  L  V  S  F  I  Q  I  M  A  T  I  F  E  W  M  Y  V  P  R  R  W  A  G  A  Q

2401  CATCTTACCAAACGCCTGCTCTTCTTGCTCTTGGTTTTCGTCATCAACCTGGCCCCTGGTGTTGTTGTCTTCGGATTTTCATCGGTGCTTCCTGGCAAGGTTCCGCTTGTCGTTGGTATC
 559   H  L  T  K  R  L  L  F  L  L  L  V  F  V  I  N  L  A  P  G  V  V  V  F  G  F  S  S  V  L  P  G  K  V  P  L  V  V  G  I

2521  GTCCATTTCTTCATCGCCCTCGCCACATTTTTCTTCTTCTCCATCATGCCACTTGGTGGCTTATTTGGCAGTTACCTGAAGAAACACGGCCGCCAGTACGTTGCCAGTCAGACGTTCACT
 599   V  H  F  F  I  A  L  A  T  F  F  F  F  S  I  M  P  L  G  G  L  F  G  S  Y  L  K  K  H  G  R  Q  Y  V  A  S  Q  T  F  T

2641  GCCAGTTTTCCGAGGCTGAAAGGAAATGATATGTGGATGTCATATGGACTTTGGGTCTGTGTTTTTGGTGCGAAATTGGCCGAGTCATACTTCTTCTTCTTGACCTTGTCCATCAAAGATCCC
 639   A  S  F  P  R  L  K  G  N  D  M  W  M  S  Y  G  L  W  V  C  V  F  G  A  K  L  A  E  S  Y  F  F  L  T  L  S  I  K  D  P

2761  ATTCGGTACTTATCGCCATACCACGTTCACCAATGCGCGGGTGTCAAGTACATTGGAGACAAGATCTGCTACTACCAGCCCCAGATTCTTCTTGGACTAATGTTCTTTATGGATCTGACG
 679   I  R  Y  L  S  P  Y  H  V  H  Q  C  A  G  V  K  Y  I  G  D  K  I  C  Y  Y  Q  P  Q  I  L  L  G  L  M  F  F  M  D  L  T

2881  CTCTTCTTCCTCGACAGTTATCTCTGGTACATTATTTGCAACACCATTTTCTCAGTCGCTAGATCGTTCTACCTTGGTGTCTCTATTTGGTCTCCCTGGCGAAACATCTTTTCGCGTCTC
 719   L  F  F  L  D  S  Y  L  W  Y  I  I  C  N  T  I  F  S  V  A  R  S  F  Y  L  G  V  S  I  W  S  P  W  R  N  I  F  S  R  L

3001  CCGAAACGTATCTACTCAAAAGTCCTTGCGACCACGGATATGGAGATCAAGTACAAGCCAAAGGTTTTGATCTCACAAGTTTGGAACGCAATAATTATCTCCATGTACAGGGAGCACCTT
 759   P  K  R  I  Y  S  K  V  L  A  T  T  D  M  E  I  K  Y  K  P  K  V  L  I  S  Q  V  W  N  A  I  I  I  S  M  Y  R  E  H  L

3121  CTCGCCATCGACCACGTCCAGAAGCTACTCTATCATCAAGTGCCGTCCGAGCAAGAAGGCAAGCGAACCCTTCGAGCACCCACCTTCTTCGTTTCCCAGGAAGACCAGTCATTCAAGACT
 799   L  A  I  D  H  V  Q  K  L  L  Y  H  Q  V  P  S  E  Q  E  G  K  R  T  L  R  A  P  T  F  F  V  S  Q  E  D  Q  S  F  K  T

3241  GAATTTTTCCCACGTGGTAGCGAAGCAGAGCGCCGTATTTCGTTCTTTGCCCAGTCGCTGTCCACCCCGATGCCTGAGCCGCTGCCTGTTGACAACATGCCTACCTTCACCGTTTTGATT
 839   E  F  F  P  R  G  S  E  A  E  R  R  I  S  F  F  A  Q  S  L  S  T  P  M  P  E  P  L  P  V  D  N  M  P  T  F  T  V  L  I

3361  CCTCATTACAGCGAAAAGATCCTTCTCTCTTTGCGTGAGATCATTCGCGAGGATGAGCCTTACTCCCGTGTGACTCTCCTGGAGTACTTGAAGCAGCTTCATCCCCATGAGTGGGACTGT
 879   P  H  Y  S  E  K  I  L  L  S  L  R  E  I  I  R  E  D  E  P  Y  S  R  V  T  L  L  E  Y  L  K  Q  L  H  P  H  E  W  D  C

3481  TTCGTTAAGGACACTAAGATTCTGGCGGACGAGACCTCTCAGTTCAATGGAGACTACGAAAAGAACGAGAAGGACGCGGCCAAGAGCAAGATTGATGACTTGCCATTCTACTGTATCGGT
 919   F  V  K  D  T  K  I  L  A  D  E  T  S  Q  F  N  G  D  Y  E  K  N  E  K  D  A  A  K  S  K  I  D  D  L  P  F  Y  C  I  G

3601  TTCAAGTCTGCTGCTCCCGAGTACACGCTTCGCACCCGTATTTGGTCCTCGCTGCGTTCGCAAACTCTTTACAGAACTGTATCCGGGATGATGAACTATAGCAGAGCTATCAAGCTCCTC
 959   F  K  S  A  A  P  E  Y  T  L  R  T  R  I  W  S  S  L  R  S  Q  T  L  Y  R  T  V  S  G  M  M  N  Y  S  R  A  I  K  L  L
```

FIG. 2. Nucleotide and predicted peptide sequences of the *fksA* gene from *A. nidulans*. Potential TATA boxes and 5′ and 3′ internal intron consensus sequences are underlined.

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3721  TACCGTGTGGAGAACCCGGAAGTCGTCCAGATGTTCGGTGGTAATTCTGAGAAGCTGGAACATGAGCTCGAGAGGATGGCCCGTCGCAAGTTCAAGATCTGTGTTTCAATGCAGCGGTAT
 999  Y R V E N P E V V Q M F G G N S E K L E H E L E R M A R R K F K I C V S M Q R Y

3841  GCCAAATTCACAAAAGAAGAACGTGAGAACACAGAGTTCCTCCTCCGAGCCTACCCCGACCTGCAGATTGCCTATCTCGATGAGGAACCTCCAGCCAACGAGGGTGAAGAGCCGCGTCTC
1039  A K F T K E E R E N T E F L L R A Y P D L Q I A Y L D E E P P A N E G E E P R L

3961  TACTCTGCTTTGATTGATGGACACTGTGAGCTGCTCGAGAATGGCATGCGGAAGCCCAAGTTCAGGATCCAGCTCTCCGGAAACCCGATCCTTGGTGACGGCAAGTCTGACAACCAAAAC
1079  Y S A L I D G H C E L L E N G M R K P K F R I Q L S G N P I L G D G K S D N Q N

4081  CACTCGATCATTTTCTACCGCGGTGAATACATTCAGGTCATTGATGCCAACCAAGACAACTATCTCGAAGAGTGCTTGAAAATCCGAAGCGTTCTTGCTGAGTTTGAGGAATTGACCACC
1119  H S I I F Y R G E Y I Q V I D A N Q D N Y L E E C L K I R S V L A E F E E L T T

4201  GACAATGTCTCGCCTTACACTCCTGGCGTTGCCTCTTCCTCTGAAGCTCCTGTTGCTATCCTTGGTGCCCGTGAATACATTTTCTCAGAGAACATTGGTGTACTTGGTGACGTTGCCGCC
1159  D N V S P Y T P G V A S S S E A P V A I L G A R E Y I F S E N I G V L G D V A A

4321  GGTAAAGAACAGACATTTGGTACCCTGTTTGCTCGTACTCTTGCTCAGATTGGCGGAAAGCTCCATTATGGTCACCCTGATTTCCTGAATGGTATCTTCATGACTACCAGAGGTGGTATC
1199  G K E Q T F G T L F A R T L A Q I G G K L H Y G H P D F L N G I F M T T R G G I

4441  TCCAAGGCTCAAAAAGGTCTACACCTTAACGAGGATATCTACGCTGGTATGAACGCCATGGTTCGTGGTGGCCGCATCAAGCACTGCGAGTACTTCCAGTGTGGTAAGGGTCGTGATCTT
1239  S K A Q K G L H L N E D I Y A G M N A M V R G G R I K H C E Y F Q C G K G R D L

4561  GGTTTCGGTTCCATTCTTAATTTCACCACTAAGATTGGCACTGGTATGGGTGAGCAAATGCTATCAAGAGAGTACTACTACTTGGGTACTCAACTGCCACTCGACCGATTCCTGTCCTTT
1279  G F G S I L N F T T K I G T G M G E Q M L S R E Y Y Y L G T Q L P L D R F L S F

4681  TACTATGCTCACCCTGGATTCCACATCAACAACATGTTTATTATGTTGTCTGTGCAAATGTTCATGATTGTTCTGATCAACCTGGGGGCCCTGAAGCACGAAACCATCAACTGCAACTAC
1319  Y Y A H P G F H I N N M F I M L S V Q M F M I V L I N L G A L K H E T I N C N Y

4801  AACTCCGACCTGCCCATTACCGATCCACTTATGCCAACGTTCTGCGCGCCTCTCACTCCTATCATCAACTGGGTCAACCGCTGTGTTATTTCGATTTTCATCGTTTTCTTCATTTCGTTT
1359  N S D L P I T D P L M P T F C A P L T P I I N W V N R C V I S I F I V F F I S F

4921  GTTCCTTTGGCTGTTCAAGAATTGACTGAAAGAGGACTCTGGCGTATGGCAACGCGTCTGGCCAAACATTTCGGATCTTTCTCCTTCATGTTCGAGGTGTTTGTTTGTCAAATCTATTCC
1399  V P L A V Q E L T E R G L W R M A T R L A K H F G S F S F M F E V F V C Q I Y S

5041  AACGCTGTGCACCAAAACTTGTCTTTCGGTGGAGCGCGCTACATCGCTACCGGTCGTGGTTTCGCAACTGCTCGTATCCCATTCGGCGTTCTGTACTCTCGGTTTGCGGGACCTTCAATT
1439  N A V H Q N L S F G G A R Y I A T G R G F A T A R I P F G V L Y S R F A G P S I

5161  TACACCGGTTTCCGTCTGCTGATCATGCTGCTCTTCTCAACCTCAACTACCTGGACTGCCTCTCTCATTTGGTTCTGGGTCTCTCTTCTCGCCCTTTGCATCTCCCCATTCCTTTTCAAC
1479  Y T G F R L L I M L L F S T S T T W T A S L I W F W V S L L A L C I S P F L F N

5281  CCTCACCAGTTTGCCTGGAACGACTTCTTCATCGATTACCGTGACTACATCCGATGGCTTTCGCGCGGTAACTCTCGCTCACACGCATCCTCATGGATTGGCTTCTGCCGTTTGTCGCGT
1519  P H Q F A W N D F F I D Y R D Y I R W L S R G N S R S H A S S W I G F C R L S R

5401  ACTCGGATCACTGGTTACAAGCGCAAGCTTCTCGGTGTGCCGTCGGAGAAAGGATCAGGTGACGTTCCCAGAGCTCGTATTACCAACATTTTCTTCAGCGAAATTGTCGCTCCTCTAGTC
1559  T R I T G Y K R K L L G V P S E K G S G D V P R A R I T N I F F S E I V A P L V

5521  CTCGTTGCTGTTACCCTCGTTCCATACCTCTACATCAATTCTCGGACTGGTGTGAGCGCTGATGTGGACGGGGGCAATGACCCTCACGATGCCATTTTGCGTATTGCCATTGTAGCATTT
1599  L V A V T L V P Y L Y I N S R T G V S A D V D G G N D P H D A I L R I A I V A F

5641  GGACCTATTGGTATCAATGCCGGTGTTGCTGCTGTTTTCTTTGGTATGGCATGCTGCATGGGTCCCATCCTGAGCATGTGCTGCAAGAAGTTCGGTGCTGTGTTGGCGGCTATTGCCCAC
1639  G P I G I N A G V A A V F F G M A C C M G P I L S M C C K K F G A V L A A I A H

5761  GCGATTGCTGTGATCATCTTGCTTGTCATCTTTGAAGTCATGTTCTTCCTCGAACACTGGTCTTGGCCCCGGTGCGTCATGGGCATGATCGCCATGGCTGCCATTCAACGTTTCGTCTAC
1679  A I A V I I L L V I F E V M F F L E H W S W P R C V M G M I A M A A I Q R F V Y

5881  AAACTTATTATCGCGCTCGCTCTTACCCGAGAGTTCAAGCATGACCAGTCGAACATCGCATGGTGGACTGGAAAATGGTACAACATGGGTTGGCACTCTCTCTCTCAACCGGGCCGAGAG
1719  K L I I A L A L T R E F K H D Q S N I A W W T G K W Y N M G W H S L S Q P G R E

6001  TTCCTCTGCAAGATCACGGAGTTGGGCTATTTCTCAGCAGACTTCGTCATTGGTCATCTCCTATTGTTCATTATGCTGCCCGCTCTTTGTGTTCCTTACATTGACAAGTTTCACTCAGTC
1759  F L C K I T E L G Y F S A D F V I G H L L L F I M L P A L C V P Y I D K F H S V

6121  ATTCTCTTTTGGCTCCGCCCAAGGTAAGACCCCTCATTTACTCGGTTGTGGATTCCTAATCTCTAACACTCAAATCTAGTCGGCAAATTCGCCCTCCAATCTACTCGCTTAAGCAGTCCA
1799  I L F W L R P S                                                 R Q I R P P I Y S L K Q S K

6241  AGCTTCGTAAGCGACGTGTGGTCCGTTTCGCTATTCTGTATTTCGCCATGCTTATCCTGTTCCTCGTTCTCCTTATTGCACCCCTCATTGCCAGAAATCAGGATCTGAACCTGAAAATGA
1821    L R K R R V V R F A I L Y F A M L I L F L V L L I A P L I A R N Q D L N L K M S

6361  GTGGCATCCTTCTTGAGCTCATGCAGCCTCTCGACAGCAACAACAACGACACTATGACCAGCTATACCGGCTCGGGTGTCCCCAAGGGCATGGAGCCTATTGCGTCCCCGTCATCGTACC
1861    G I L L E L M Q P L D S N N N D T M T S Y T G S G V P K G M E P I A S P S S Y L

6481  TTACGAACTTAAACAACTAAAGGCTGTCTTGTTTCGTCAAACTACATTCTGCTACAATATTCTGATATGGAACATGGGAACAAGATGTATAAAGTATCCGGCGCGTTGATGTTGCTGTTG
1901    T N L N N

6601  AGCCCGTCAATTATGTTTCTTATTTCCATATTATTCTCCTCAACGGAATCTCTACTTGTACGGTTCCTGTGAGATTCAATGGATGCAATATCTATCGGCTTGTTTT
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FIG. 2—*Continued.*

start site (underlined), TCATAATGTC, is nearly identical to the consensus T(C)C(A)ACAATGTC reported by Ballance that was derived from the sequence of 12 *Aspergillus* genes (6).

Comparison of predicted FksAp to *S. cerevisiae* Fksps. The predicted *fksA* peptide (FksAp) was compared with *S. cerevisiae* Fks1p and Fks2p and with the predicted product of an open reading frame on chromosome XIII of *S. cerevisiae* designated a homolog of *FKS1* (42). We refer to this homolog as Fks3p, although the function of Fks3p is not yet known. FksAp is slightly larger than the other Fksps, with most of the additional amino acids located in the N terminus (multiple se-

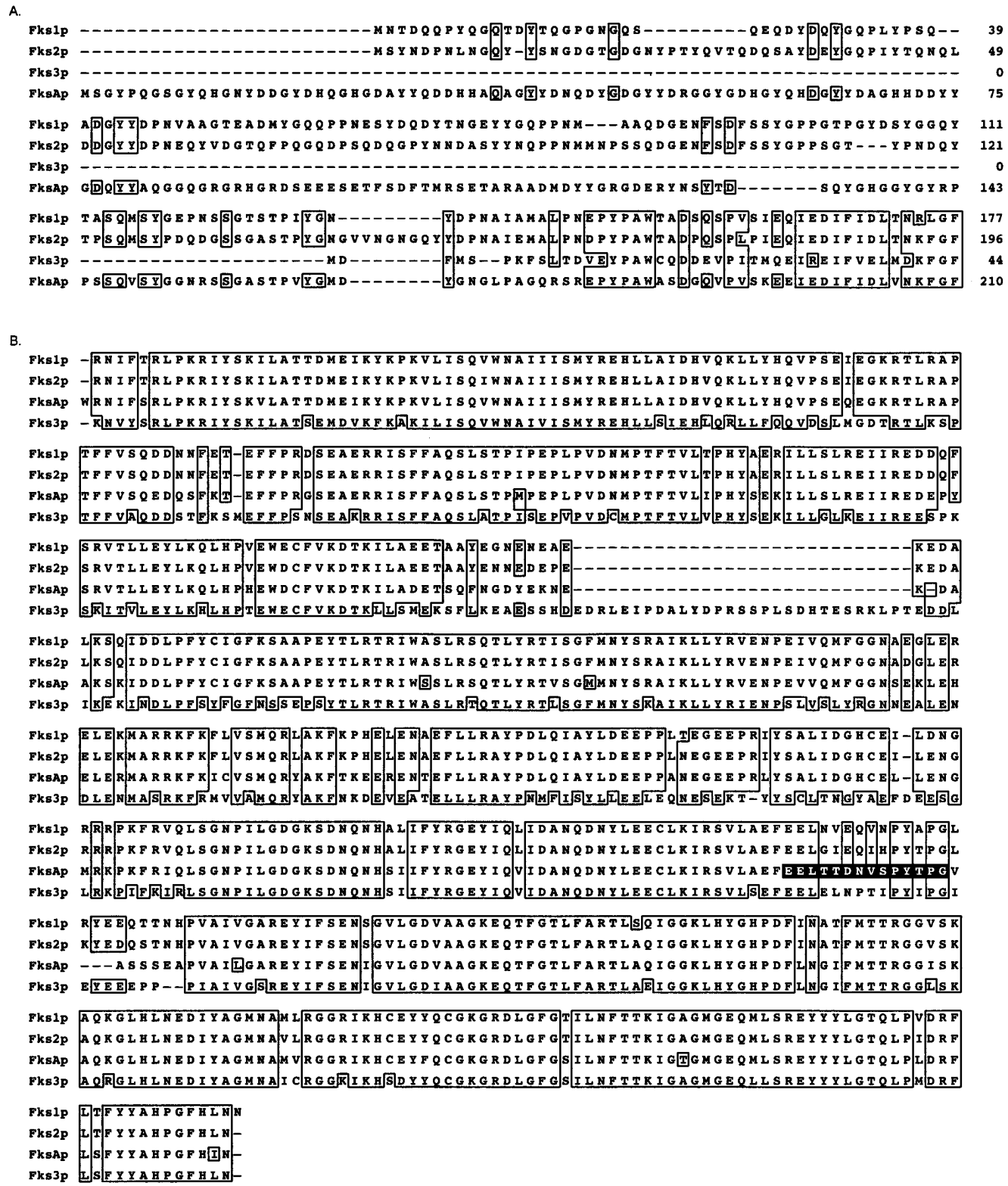

FIG. 3. Multiple sequence alignments of FksAp, Fks1p (accession number U12893), Fks2p (accession number U16783), and Fks3p (accession number Z49212). (A) Alignment of the amino terminal 210 amino acids of FksAp with the corresponding region of the other proteins. Alignment was performed with the PileUp Program, which uses a simplification of a progressive alignment method (25). (B) Alignment of large hydrophilic domains hypothesized to contain the catalytic site. These domains were determined from the hydropathy and membrane topology data presented in Fig. 4 and correspond to amino acids 751 to 1328 of FksAp, 720 to 1301 of Fks1p, 739 to 1319 of Fks2p, and 605 to 1213 of Fks3p. The 14-amino-acid sequence of FksAp which was used to raise antisera is highlighted.

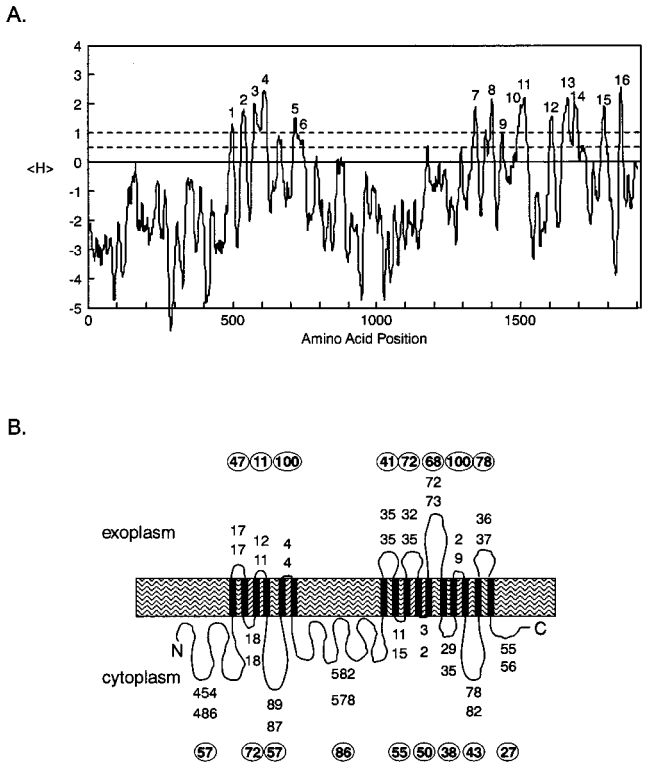

FIG. 4. Hydropathy analysis of FksAp. (A) Hydropathy profile of FksAp by the method of Sipos and von Heijne (56). A full window of 21 amino acids and a core window of 11 amino acids were used. The upper dotted line with a mean hydropathy value (<H>) of 1.0 designates the lower limit for transmembrane regions predicted with a high degree of certainty. The other dotted line with <H> of 0.5 indicates the lower limit for putative transmembrane domains. The numbers above each peak correspond to the predicted transmembrane domains. (B) Model comparing the predicted membrane topologies of FksAp and Fks1p. The membrane is shown as a rectangle containing wavy lines, and the solid black line designates the polypeptide chain. The 16 predicted 21-amino-acid transmembrane helices are indicated by vertical black bars. The length (number of amino acids) of each nontransmembrane domain is shown; the inner numbers designate Fks1p, and outer numbers designate FksAp. The percent identity between the corresponding nontransmembrane domains of FksAp and Fks1p is shown in the circle.

quence alignment is shown in Fig. 3A). The isoelectric point of the predicted FksAp is 7.92, which is also similar to the pIs of the other homologs.

FksAp is 64 and 65% identical to Fks1p and Fks2p, respectively, and 57% identical to Fks3p. The least conserved regions are the N and C termini. The N terminus of FksAp (amino acids 1 to 166) exhibits only 28 and 23% identity to the N termini of Fks1p (amino acids 1 to 133) and Fks2p (amino acids 1 to 152), respectively. The N termini of Fks1p and Fks2p also diverge significantly, with 48% identity, compared with 88% identity overall, but both are acidic and have similar unusual amino acid compositions (42). The N terminus of FksAp is also acidic (pI of 4.41) and has a preponderance of glutamine and glycine residues as was noted previously for Fks1p and Fks2p. Interestingly, Fks3p lacks this corresponding N-terminal region; instead, the beginning of the protein aligns with amino acid 167 of FksAp (Fig. 3A). The C terminus of FksAp is 22 and 25% identical to Fks1p and Fks2p, respectively, and 38% identical to Fks3p.

The hydropathy profile of FksAp is shown in Fig. 4A. To assess the topology, all of the certain and putative transmembrane helices predicted for FksAp, Fks1p, and Fks2p were localized on a multiple sequence alignment. All 16 of the predicted transmembrane helices of FksAp coincide with the transmembrane helices of Fks1p and Fks2p, but several are shifted slightly relative to the yeast homologs. The numbers of amino acids in the nontransmembrane domains of FksAp and Fks1p are strikingly similar (Fig. 4B), and both N termini are predicted to be cytoplasmic (18, 30). The large hydrophilic cytoplasmic domain of 578 amino acids is the region most homologous to the *S. cerevisiae* proteins, exhibiting 86% identity (Fig. 3B). The corresponding nontransmembrane domain of Fks3p is somewhat larger, 609 amino acids, and is 72% identical to the 578-amino-acid domain of FksAp.

A search of the protein database with the entire FksAp sequence by using BLASTP (2) did not reveal any proteins with significant homology in addition to the *S. cerevisiae* homologs. The highly conserved hydrophilic domain of 578 amino acids (FksAp578) is a good candidate for the catalytic site and was used to perform a similar search. The catalytic subunit of bacterial cellulose synthase of *Acetobacter xylinum* (accession number SP 19449) (39, 51, 60), encoded by the gene *bcsA*, was identified with a probability score of 0.19, where 1.0 is chance. Cellulose synthase catalyzes the formation of β-1,4-glucan, using UDPG as a substrate. Two of the three segments identified by the search, designated domains 1 and 2, are shown in Fig. 5. Domain 1 is 25 amino acids long, exhibiting 44% identity and 64% similarity, while domain 2 contains 37 amino acids with 29% identity and 56% similarity. Similar conservation was found between these two domains of BcsAp and those of Fks1p and Fks2p.

Biochemical characterization of GS activity. Solubilization of GS was accomplished by extraction with the nonionic detergent W1. The apparent K_m for UDPG (2 to 2.5 mM) and the V_{max} (6 to 10 nmol/min/mg of detergent-extracted protein) were determined from double-reciprocal plots of $1/S$ versus $1/V$ during the linear incorporation phase at each substrate level. Extraction activated the enzyme two- to threefold without changing the apparent K_m for UDPG or the IC_{50} for L-733,560 (Table 1).

A. nidulans GS, like those of other fungi, is activated by guanine nucleotides (33). Extraction did not significantly shift the activation curve for nucleotides. Ten micromolar GTPγS was saturating in assays of both particulate and detergent-extracted membranes isolated without any added guanine nucleotide, stimulating particulate GS by about 10-fold and solubilized GS by about 20-fold. One to 2 μM GTPγS gave half-maximal stimulation in both cases. The pH optimum of 7.5 was also unaffected by detergent extraction. The enzyme product was characterized as described by Douglas et al. (19) and found to be identical in size (degree of polymerization of about 1500) with that of the *Saccharomyces* GS product. The high-molecular-weight *A. nidulans* product was more than 95% digested by laminarinase.

Domain 1

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FksAp   861 PMPEPLPVDNMPTFTVLIPHYSEKI 885
            P+P P  VD+ PT  + IP Y E++
BcsAp   138 PLPLPDNVDDWPTVDIFIPTYDEQL 162
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Domain 2

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FksAp   968 LRTRIWSSLRSQTLYRTVSGMMNYSRAIKLLYRVENP 1004
            LR  + S L ++ L   +   M ++R +  ++RV+NP
BcsAp   350 LRIPVASGLATERLTTHIGQRMRWARGMIQIFRVDNP 386
```

FIG. 5. Domains 1 and 2 are conserved regions between FksAp and BcsAp. Shown are the actual high-scoring segment pairs detected in a BLASTP search with FksAp578 as the query sequence. + indicates similar amino acids.

TABLE 1. Purification scheme[a]

Fraction	Vol (ml)	Protein (mg/ml)	Sp act (U/mg)[b]	Activity (U)	Yield (%)	L-733,560 IC_{50} (nM)
Crude membrane	18	5	3.3	297	100	8
Depleted W1	17	3.5	11.6	690	232	8
Residual pellet	0.75	0.20	490	73	25	ND[c]
Solubilized PE enzyme	2.2	0.028	850	62	21[d]	1

[a] Ninety-milligram aliquots of washed particulate membranes were extracted with 0.025% NP-40 and then with 0.3% W1 to yield depleted W1 extract (described in Materials and Methods) and subjected to one round of product entrapment and subsequent solubilization, giving rise to the solubilized PE enzyme and a residual pellet. Assays were performed at 2 mM UDPG in the presence of 10 μM GTPγS.

[b] One unit of activity is defined as 1 nmol of glucose incorporated per min of incubation at 22°C.

[c] ND, not determined.

[d] The cumulative yield of enzyme released by three extractions of the PE pellet with 0.1% W1. Assays of the PE enzyme were performed at final concentrations of 0.01% W1 and 0.05% NP-40.

Conventional purification methods gave very low yields of GS and little enrichment. Product entrapment however, afforded a high degree of purification when refined for working at high protein concentrations and when a preextraction step was added prior to solubilization of GS as described in Materials and Methods. Although W1 efficiently solubilized *Aspergillus* GS, it reduced enzyme activity by 90% at a final concentration of 0.1%. The inhibition by W1 was relieved by the nonionic detergent NP-40, optimally at an NP-40/W1 ratio of 5:1. This discovery allowed the use of W1 levels sufficient to extract GS activity from 5 mg of membranes per ml in trapping reactions. A typical purification is shown in Table 1. Similar results were obtained from three independent purifications. One preparation resulting from a single extraction of the PE enzyme provided GS with our highest specific activity (1,500 U/min/mg), with an overall yield of 9%. The addition of fraction A in saturating amounts stimulated enzymatic activity by about 50%, suggesting that purification by entrapment partially depleted GS of the GTP-activated subunit, even in the presence of nonhydrolyzable guanine nucleotide.

Silver staining of the soluble enzyme from the purification in Table 1 is shown in Fig. 6. There are proteins at just less than 200 kDa and at 110, 55, 31, and at 20 kDa. All preparations, including our purest with a specific activity of 1.5 μmol/min/mg, consistently show these proteins.

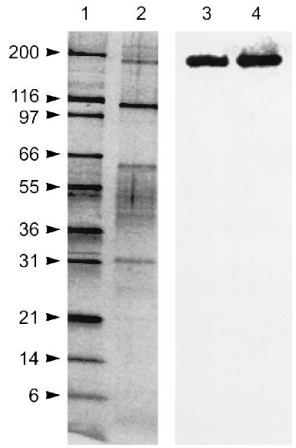

FIG. 6. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of PE GS. Lanes 1 and 2, silver staining performed with a two-dimensional silver stain kit (Daiichi); lane 1, standards (Bio-Rad Mark 12); lane 2, 0.5 μg of the solubilized pellet after one round of entrapment; lanes 3 and 4, Western blot analysis performed with anti-FksAp antiserum (1:5,000 dilution) and goat anti-rabbit antibody-horseradish peroxidase conjugate as the second antibody, followed by detection by NEN Renaissance amplification; lane 3, 5 μg of crude W1 extract; lane 4, 0.1 μg of solubilized PE GS. Samples were dissolved in reducing, denaturing 2× electrophoresis buffer at room temperature (without heating) prior to electrophoresis on a 4 to 20% SDS-Tris-glycine gel (Novex).

Antisera against a synthetic FksAp peptide strongly reacted with a protein in W1 extracts migrating just below the 200-kDa standard (Fig. 6). The antiserum also reacted to a protein at the same position in crude membranes and in B fractions and was almost completely absent in the soluble A fraction (data not shown), as expected for an integral membrane protein involved in enzymatic activity. The 200-kDa signal was enriched by product entrapment approximately in proportion to specific activity (Fig. 6, lane 4, which contains 50-fold less protein than lane 3). The signal at 200 kDa was fully blocked when 1 μM *Aspergillus*-specific peptide was present in the primary antibody adsorption step but was not blocked by the one yeast *FKS1*-derived or two *FKS2*-derived 14-mer peptides described by Mazur et al. (42). Antisera raised to these yeast peptides did not recognize FksAp, and conversely the anti-FksAp antibodies did not recognize *Saccharomyces* Fks1p or Fks2p, which differ by 5 and 4 amino acids out of 14, respectively (Fig. 3B). No antisera to entirely conserved regions were made.

Highly enriched GS was inhibited by L-733,560, with an IC_{50} of 1 nM. This is very close to the minimal effective concentration (1.7 nM) and is lower than the IC_{50} of 8 nM obtained with particulate membranes or crude detergent extracts.

Immunoprecipitation. The FksAp antiserum neither reduced enzyme activity nor yielded immunoprecipitate. However, FksAp antiserum immunodepleted activity in the presence of *Staphylococcus aureus* cells (Pansorbin; Calbiochem) which precipitate immunoglobulin G (IgG). Typical results with affinity-purified IgG are shown in Fig. 7, using crude solubilized (W1) extract and solubilized PE enzyme. Nearly complete depletion of enzymatic activity from both crude and purified GS was obtained with 4 μg of IgG (Fig. 7A). Recovery of activity from the resuspended *Staphylococcus aureus* pellet was low in crude extracts (10 to 20%) but was higher (50 to 60%) in purified GS (Fig. 7B). This difference may be due to proteolytic contaminants in the initial detergent extract or to more severe loss of components other than A fraction in the crude system compared with the purified. Adding back the supernatant of the immunodepletion reaction did not significantly raise recoveries from either the crude or purified immunoprecipitated pellets. The presence of competing peptide reduced immunoprecipitation, almost fully eliminating recovery of activity in the pellet for purified GS (Fig. 7B). The reason for the low level of enzymatic activity remaining in the supernatant in the presence of peptide (Fig. 7A) is unclear, since peptide alone had little effect on enzymatic activity.

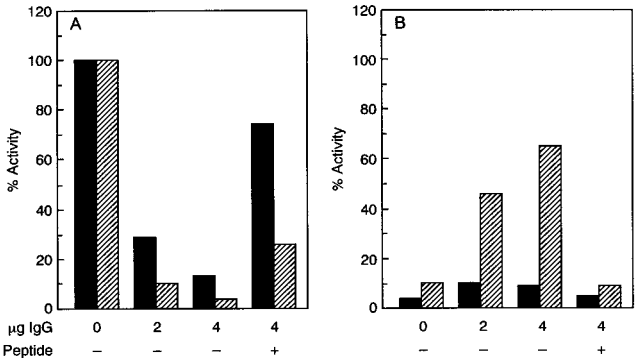

FIG. 7. Immunoprecipitation of GS activity. (A) Depletion of activity from W1 extract (solid bars) and solubilized PE GS (hatched bars). Approximately 0.1 U (nmol/min) of GS was used in both cases. (B) Recovery of activity from the cell pellet following immunoprecipitation. After three washes of the pellet with PBS–1% NP-40, the cells were resuspended in 25 μl of 25 mM HEPES (pH 7.5)–1% NP-40, and duplicate 10-μl aliquots were assayed after the addition of 2 μg of fraction A. A 14-mer peptide (1 μM) was included in the incubation with antiserum as shown.

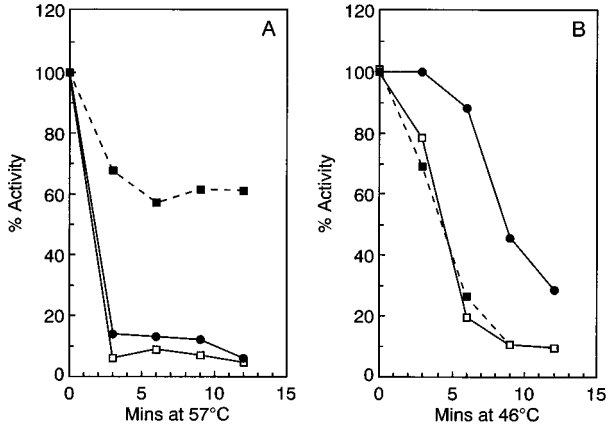

FIG. 8. Protection of separated components from heat denaturation. (A) Fraction A. GTPγS (50 μM), filled squares; UDPG (10 mM), filled circles; no addition, open squares. Duplicate aliquots of fraction A (0.66 mg/ml in extraction buffer containing 0.025% NP-40) were exposed to 57°C with or without supplements as shown for the indicated times and added to B fraction kept at 4°C prior to assay at 22°C under the usual conditions. (B) Fraction B. UDPG (10 mM), filled circles; GTPγS (50 μM), filled squares; no addition, open squares. Aliquots of fraction B (1.37 mg/ml in 0.05M KPO_4 buffer [pH 7.5] containing 0.02% NP-40) were heated at 46°C and added to unheated fraction A prior to assay.

Heat lability of the separated fractions. GSs from several fungi have been separated into a membrane-associated and readily solubilized A fraction and an integral membrane particulate B fraction (33). Each has low GS activity when assayed alone but substantial activity when assayed together. We prepared a soluble B fraction by gel filtration of detergent-solubilized GS. The protein eluting in the void volume contained FksAp, as demonstrated by Western blotting (results not shown), and was nearly devoid of GS activity. Reconstitution with A fraction activated the soluble B fraction 10- to 15-fold, restoring GS activity to approximately that of crude membranes (Table 2). Sensitivity to L-733,560 in the reconstituted *A. nidulans* system was identical to that of crude membranes, demonstrating that no loss of drug target had occurred during the fractionation.

Fraction B is very labile at temperatures above 40°C. Heat lability of this fraction was unaltered by the presence of either low levels (1 to 5 nM) of L-733,560 or by laminaroligosaccharides. It was significantly protected from inactivation by 10 mM UDPG but not by GTPγS (Fig. 8B). The A fraction was less labile, requiring temperatures above about 55°C for appreciable inactivation; protection in this case was provided by 50 μM GTPγS but not by UDPG (Fig. 8A). The pattern of protection against heat inactivation suggests that the catalytic subunit is localized in the B fraction and the GTP-binding subunit is localized in the A fraction.

TABLE 2. Fractionation of GS into soluble complementary components[a]

Fraction	Protein (mg)	Sp act (U/mg)	Activity (U)	Yield (%)
Crude membrane	10	3.5	35	100
W1[b]	6.4	9.5	61	174
Fraction A[c]	1.6	0.5	0.8	2
Depleted W1[d]	4.9	8.0	39	112
Fraction B[e]	3.6	0.6	2.2	6
Fraction A + fraction B	5.2	6.2	32	92

[a] Results are shown for one experiment. Similar results were obtained in two additional fractionations.
[b] Supernatant from direct extraction of crude membranes by W1 detergent.
[c] Supernatant from extraction of crude membranes with 0.025% NP-40.
[d] Supernatant resulting from W1 extraction of the residual pellet referred to in footnote *c*.
[e] Soluble protein eluting in void volume after gel filtration of depleted W1 extract.

DISCUSSION

Several lines of evidence suggest that the cloned *fksA* gene encodes a subunit of GS. First, antibody derived from the *fksA* sequence recognizes in Western blot analysis a protein of ~200 kDa, close to the predicted size of FksAp, that is enriched in purified preparations of GS from *A. nidulans* in proportion to the increase in specific activity. Further, the antibody can be used to deplete crude and purified enzyme preparations of GS activity, with partial recovery of the depleted activity in the corresponding pellets. Second, the most highly purified preparations of GS, enriched over 300-fold, are inhibited by the pneumocandin L-733,560 at concentrations that cause morphological alterations in whole cells. Third, fractionation of solubilized GS activity into two complementary fractions shows that FksAp is localized in the membrane-associated fraction, a location consistent with its sequence which contains many membrane-spanning regions. FksAp is at the very least a protein which copurifies with the catalytic subunit of GS over a significant range of purification, without appreciable loss relative to catalytic activity.

The topology of FksAp predicted from its sequence is that of a membrane protein composed of two large hydrophobic domains containing several transmembrane helices separated by a large cytoplasmic hydrophilic domain. This structure resembles that of some bacterial and eukaryotic transport proteins (49). A protein with the membrane topology described for FksAp might be a pore involved in the export of nascent glucan chains or coiled fibrils through the plasma membrane. If this is its role, rather than being the catalytic subunit itself, FksAp must retain high affinity for the solubilized complex containing the catalytic apparatus even after solubilization into detergent micelles and extensive purification. Our data showing that the solubilized fraction containing FksAp is protected from heat inactivation by the substrate UDPG support the colocalization of FksAp and the catalytic subunit. The simplest explanation of

our data for *A. nidulans*, and analogous data for *S. cerevisiae* (42), is that Fksps are themselves catalytic subunits.

The pattern of proteins that we obtained in preparations of GS purified by product entrapment is similar to that described for *N. crassa* and *S. cerevisiae* (4, 31). The 165-kDa *N. crassa* protein and the 200-kDa *S. cerevisiae* protein were specifically labeled with the affinity substrate azido-UDPG, and competition for labeling was demonstrated with appropriate nucleotides (4, 59). The 165-kDa protein presumably represents the corresponding *FKS* homolog in *N. crassa*. The 200-kDa protein migrates at precisely the same position as *S. cerevisiae* Fks1p and Fks2p detected by Western blotting (31, 42). We were unable to label FksAp specifically with azido-UDPG.

It is intriguing to speculate that some of the proteins that copurify with the ~200-kDa *A. nidulans* protein are additional subunits of GS or other proteins important for GS activity (44). Rho1p, a protein of 23 kDa, has recently been identified as the GTP-binding subunit of GS in *S. cerevisiae* (20, 46). The Rho1p homolog in *A. nidulans*, identified as a C3 toxin-ribosylatable protein at ~20 kDa (41), may be the 20-kDa protein in our preparations. The prominent protein at about 110 kDa (Fig. 6) is also observed in similar preparations from *S. cerevisiae* and *N. crassa* (4, 31). The *S. cerevisiae* protein has been identified as the plasma membrane H^+-ATPase (encoded by *PMA1*) by amino acid sequencing of three tryptic peptides from the purified protein (5). Polyclonal antibodies raised against the purified *Saccharomyces* 110-kDa protein cross-react with the *A. nidulans* 110-kDa protein in Western blot analysis (5).

The K_m for *A. nidulans* preparations of 2 to 2.5 mM UDPG closely resembles the value of 1.9 mM reported for *A. fumigatus* preparations by Beauvais et al. (8) and is about five times higher than the value of 400 μM determined by Beaulieu et al. (7). The V_{max} of 6 to 10 nmol/min/mg for *A. nidulans* is very similar to the value determined for *A. fumigatus.*

The striking similarities in size, charge, amino acid sequence, and membrane topology between FksAp and *S. cerevisiae* Fks1p and Fks2p strongly suggest that we have cloned a gene encoding a functionally equivalent protein from *A. nidulans*. FksAp resembles the expressed proteins Fks1p and Fks2p more closely than the third *S. cerevisiae* homolog, Fks3p, which is not yet known to be expressed. FksAp exhibits similar levels of identity to both Fks1p and Fks2p and may be the major Fksp in *A. nidulans*. We have been unable to detect another homolog by Southern blot analysis under hybridization conditions that led to the detection of *FKS2* in *S. cerevisiae* (data not shown). Two *FKS* sequences that exhibit only 53% identity to one another over a stretch of 137 amino acids (only partial DNA sequence has been reported for both sequences [43, 52]) have been identified in *C. albicans*. Thus, it is possible that an Fksp with less homology to FksAp is also present in *A. nidulans.*

Gene disruption of *fksA* should prove conclusively that it encodes a functional protein, and it will be interesting to see if this a lethal event. We believe that 1,3-β-glucan is essential for viability of *A. nidulans* because of the susceptibility of hyphal growth to pneumocandins, although the cell wall of *A. nidulans* is composed of less 1,3-β-glucan and more chitin than the cell wall of *S. cerevisiae* (reviewed in references 17, 35, and 48). Only two mutants of *A. nidulans*, with defects in the *orlC* and *orlD* genes, have been reported to be defective in 1,3-β-glucan (10). These mutants were isolated in a screen for a temperature-sensitive, osmotically remedial lysis phenotype, and they have reduced amounts of 1,3-β-glucan but contain normal levels of GS activity (9, 45).

The identification of domains of Fksps important for catalytic activity in pathogenic fungi should be facilitated by comparison of the amino acid sequences and membrane topology of FksAp and the *Saccharomyces* Fksps. The 578-amino-acid hydrophilic domain of FksAp, which exhibits 86% identity to the corresponding regions of the *S. cerevisiae* Fksps, is a good candidate for the catalytic domain. It is intriguing that a homology search with this domain produced a match with the catalytic subunit of cellulose synthase (encoded by *bcsA*), another enzyme that polymerizes UDPG into glucan, although the probability score of the match is not highly significant. It is interesting to speculate that the conserved domains (domains 1 and 2 [Fig. 5]) may be involved in binding UDPG. The consensus UDPG binding site -R/K-X-G-G found in glycogen synthase of several species, including *S. cerevisiae* (24, 26), is not found in the Fksps or BcsAp. Perhaps these proteins contain a different sequence for binding UDPG such as the region identified in domains 1 and 2. Comparative studies of this nature will be further enhanced when more of the *FKS* genes from *C. albicans* are sequenced and when *FKS* homologs from other fungi are cloned.

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