Aspergillus nidulans swoF Encodes an N-Myristoyl Transferase

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Polar growth is a fundamental process in filamentous fungi and is necessary for disease initiation in many pathogenic systems. Previously, swoF was identified in Aspergillus nidulans as a single-focus, temperature-sensitive (ts) mutant aberrant in both polarity establishment and polarity maintenance. The swoF gene was cloned by complementation of the ts phenotype and sequenced. The derived protein sequence had high identity with N-myristoyl transferases (NMTs) found in fungi, plants, and animals. In addition, wild-type growth at restrictive temperature was partially restored by the addition of myristic acid to the growth medium. Sequencing revealed that the mutation in swoF changes the conserved aspartic acid 369 to a tyrosine. The predicted A. nidulans SwoF protein, SwoFp, was homology modeled based on crystal structures of NMTs from Saccharomyces cerevisiae and Candida albicans. The D369Y swoF mutation is on the opposite face of the protein, distal to the myristoyl coenzyme A and peptide substrate binding sites. In wild-type NMTs, D369 appears to stabilize a structural β-strand bend through two hydrogen bonds and an ionic interaction. These stabilizing bonds are abolished in the D369Y mutant. We hypothesize that a substrate of SwoFp must be myristoylated for proper polarity establishment and maintenance. The mutation prevents the proper function of SwoFp at restrictive temperature and thus blocks polar growth.

For many filamentous fungi, two distinct growth modes are used during the process of spore germination and early development. When dormancy is broken, the spore first grows isotropically, adding new cell wall material uniformly in every direction. A switch to polarized growth soon follows, with new cell wall deposition occurring only at the growing tip, leading to elongated hyphae characteristic of filamentous fungi. In Aspergillus nidulans, the switch from isotropic to polar growth occurs just after the first round of mitosis (34). Two distinct processes are involved in the switch to polar growth: polarity establishment, i.e., choosing the spot where new material will be deposited, and polarity maintenance, i.e., the continued deposition of wall material at the growing tip.

Not surprisingly, the process of polar growth requires an intact cytoskeleton and proper vesicle transport. Pharmacological disruption of F actin prevents the polar growth of Neurospora crassa and Saprolegnia ferax (22). The actin-associated motor protein myosin has a high level of tip localization, and loss of myosin function in A. nidulans leads to large apolar cells (32, 37). Mutations in sepA, which encodes an actin-associated protein, result in abnormally large, septumless A. nidulans hyphae (20). In N. crassa, kinesin and dynein mutants grow with abnormally short, thick germ tubes (40, 41). Normal vesicle assembly is also required for polar growth. The A. nidulans α-cop-related gene sodPPC, involved in coated vesicle assembly, is essential both to establish polarity and to maintain polarity (45).

Because polar growth is coordinated with nuclear division in filamentous fungi, it is also not surprising that cell division signaling pathways are involved in polarity. A cyclin-dependent kinase mutant (phoA) and a mitogen-activated protein kinase deletion mutant (mpkA) both have abnormal germination and polarity maintenance (10). Strains with mutations in the protein phosphatase gene pphA display abnormal hyphal growth and mitotic defects (28). Temperature-sensitive (ts) mutations in N. crassa protein kinase A result in swollen cells with abnormal polarity maintenance (8). Also, in N. crassa the Ser/Thr protein kinase encoded by cot1 is necessary for polar growth (18, 47). In Penicillium marneffei, the Cdc42 (Rho) homologue encoded by cflA is required for both polarity establishment and polarity maintenance (7).

The A. nidulans swo (swollen cell) mutants are characterized by either continued isotropic growth without establishment of polarity or the inability to maintain polar growth at restrictive temperatures (35). Through a series of temperature shift experiments and genetic crosses, it was determined that the events of polarity establishment and polarity maintenance are genetically separable. swoC and swoD were found to be involved in polarity establishment, while swoA was found to be required for polarity maintenance. swoF was found to be involved in both processes.

In this study, we describe the cloning, sequencing, and further characterization of swoF (35). We show that swoF encodes an N-myristoyl transferase (NMT). NMTs are highly conserved proteins that catalyze the transfer of myristate (C14:0) from myristoyl coenzyme A (myristoyl-CoA) to the N-terminal glycine of a subset of cellular proteins (4–6). This modification increases the affinity of the target protein for membranes and has been proposed to constitute a reversible mechanism that allows the target protein to switch between membrane-bounded and cytoplasmic states (5). Known targets of NMTs include protein phosphatases, protein kinases, kinase substrates, and G protein α subunits (9).

MATERIALS AND METHODS

Strains and media used. Strain A773 (pyrG89 pyrA44 wA43) was crossed with strain AJB11 (swoF) by using standard methods (21, 25) to produce strain...
AXL19 (pyrG99 swoF). All experiments reported here used strain AXL19 as the swoF mutant and strain A773 as the wild type. Media used were as previously reported (35).

Growth of germlings and microscopic observations. Conditions for growth and preparation of germlings for observations were as previously reported (35). Microscopic observations were made by using an Axioplan microscope (Zeiss, Thornwood, N.Y.), and digital images were acquired by using an Optronics (Goleta, Calif.) digital imaging system. Images were prepared by using Photoshop 5.5 (Adobe, Mountain View, Calif.).

Complementation and plasmid recovery. A genomic library was generously provided by Greg May (University of Texas M. D. Anderson Cancer Center, Houston). This library was constructed by ligating Sau3A fragments of genomic DNA into the BamHI site of pRG3AMA1 (36). Proteolysts of AXL19 were produced and transformation was conducted by using standard A. nidulans protocols (48). Transformants were selected by assaying for restoration to pyrG prototrophy. Complementation was judged by restoration of wild-type growth at restrictive temperature (42°C). The complementing plasmid replicated autonomously (e.g., extrachromosomally) due to the AMA1 sequence (1) contained in the library vector. The complementing plasmid was recovered by transformation of Escherichia coli XLI-Blue with DNA isolated from the complemented strain and selection on ampicillin-containing medium. The restriction patterns of three recovered plasmids were compared, and those showing common insert patterns were used to retransform the swoF mutant. Two of three recovered plasmids complemented swoF and showed identical restriction patterns. One of these, p19c2, was chosen for sequencing.

Sequencing. p19c2 was transposon tagged by using a GPS-1 kit (New England Biolabs, Beverly, Mass.) and was transformed into E. coli XLI-Blue (35). Colonies representing individual randomly tagged plasmids were arrayed in a 96-well format. Plasmid preparation was carried out with an R.E.A.L. 96-well kit (Promega, Madison, Wis.). Label was incorporated by using Big Dye 2.0 (Perkin-Elmer Applied Biosystems, Boston, Mass.). Sequencing was done by outward-facing primers designed on the basis of the transposon provided with the GPS-1 kit. Unincorporated dyes were removed by using a DyeEx 96 kit (Qiagen, Valencia, Calif.). Sequencing in the 96-well format was performed with an ABI Prism 3700 robotic sequencer (Applied Biosystems Inc., Foster City, Calif.). Subsequent analysis was done with the programs Phred version 0.000925c and Phrap version 0.990319 for assembly and quality determination and Consed (16; http://www.igv.org). All sequences contained at least fourfold redundancy, with a quality rating of at least 20.

Identification of the swoF gene. The complete sequence for complementing genomic DNA was compared to sequences in the National Center for Biotechnology Information database [www.ncbi.nlm.nih.gov] by using blastX. Multiple open reading frames were found within the genomic sequence. Transposon-tagged plasmids with strategically placed transposon insertions were chosen from the plasmid array to test for complementation. Transposon-tagged plasmids were transformed into AXL19. Transformants were replica plated at permissive and restrictive temperatures. The open reading frame that, when disrupted by transposon insertion, lost the ability to restore AXL19 to wild-type growth at 42°C was identified as swoF.

Sequencing of the swoF mutant allele. Genomic DNA from AXL19 was isolated by using standard methods (39). This genomic DNA was used as a template in a PCR with the Expand High Fidelity PCR system (Roche Diagnostics, Indianapolis, Ind.). The following primers were used for PCR amplification: swoF5 BsmHI, 5-GGGATCCGATGTCAGACTCAAAAGACTC-3, and swoF3 SpeI, 5-GGAGCTCTAGCTAGAATAAAACGCCCA-3. Each primer contains a 20-mer based on the wild-type swoF sequence and a 10-mer to create a restriction enzyme recognition site. PCR products were cloned into the pGEM-T vector system (Promega), transformed into E. coli XLI-Blue, and selected on ampicillin-containing medium with blue-white selection. Clones were verified by restriction analysis. Three clones were sequenced by using the strategy described above. Sequences of all three clones were compared to the wild-type sequence to determine the mutant lesion. All three had the same base change.

Protein alignment. Sequences for orthologues of NMT were obtained from GenBank [http://www.ncbi.nlm.nih.gov]. Protein sequence alignment was carried out by using the program GeneDoc version 2.6.001 (www.psc.edu/biomed/genedoc) with default parameters and minimal manual adjustment to align sequences with secondary structural features.

Homology modeling. The model for the predicted A. nidulans SwoF protein, SwoFp, was prepared by homology modeling with the program Swiss PDB Viewer version 3.7b2 (http://www.expasy.ch/spdbv/). The templates used were the atomic structures determined by X-ray crystallography of the Saccharomyces cerevisiae NMT complexed with myrisoyl-CoA and a peptide analogue (Protein Data Bank accession number 2NMT) and the Candida albicans NMT (PDB accession number 1NMT). The structure of the S. cerevisiae NMT was used as the principal model as it represents a conformationally closed complex representative of the substrate-bound structure. Loops were built by using the Swiss PDB Viewer (in some cases, program O was used (24) because of its ability to merge coordinates and slightly better modeling capabilities. Poor side-chain orientations were identified by their high energies and corrected by using the optimize function in Swiss PDB Viewer. After all loops were built and side-chain conformations were corrected, the model was energy minimized by using steep descent and conjugate gradients until the change in energy between steps was <0.05 kJ/mol and no locally poor energies remained. The model has threading energies, conformational energies, and phi-psi plots similar to those of the original crystal structures. Thus, no attempt was made to correct geometric or energetic problem areas that appeared to carry over from the atomic structures. The nomenclature for β strands and α helices follows that used by Bhattacharjee and colleagues (35). The substrate myristoyl-CoA and the peptide analogue from 2NMT were docked by using the magic-fit option. No energy minimization was required.

Growth restoration with myristate. To ascertain if myristic acid medium amendment allowed polar growth of swoF cells at restrictive temperature, A773 and AXL19 were grown either in liquid or on solid minimal medium containing 500 µM myristate and 1% (wt/vol) Brij 58 (polyoxyethylene 20 cetyl ether) (Sigma, St. Louis, Mo.). The presence of 1% Brij 58 helps to solubilize myristate (14). To ascertain if myristic acid medium amendment allowed polar growth of swoF cells at restrictive temperature, A773 and AXL19 were grown either in liquid or on solid minimal medium containing 500 µM myristate and 1% (wt/vol) Brij 58 (polyoxyethylene 20 cetyl ether) (Sigma, St. Louis, Mo.). The presence of 1% Brij 58 helps to solubilize myristate (14). Nucleotide sequence accession number. The full genomic sequence, as well as intron locations, and the predicted protein sequence determined here have been deposited in GenBank under accession no. AY057437.

RESULTS

The swoF mutant was identified in a screen for ts mutants with defects in polar growth (35). Originally, swoF was thought to be necessary for both polarity establishment and polarity maintenance based on the failure to initiate germ tube growth at a restrictive temperature and upon a shift from a restrictive to a permissive temperature. However, in our current experiments, we found that swoF cells did sometimes initiate polar growth after 16 h of incubation at restrictive temperature (42°C), making short, thick germ tubes with swollen apices (16%; n = 500) (Fig. 1b). However, the majority of swoF cells failed to make germ tubes at the restrictive temperature, as previously reported (Fig. 1d). Both swoF cells with short germ tubes and those without germ tubes completed two or three mitotic divisions after 16 h of incubation at the restrictive temperature. In contrast, wild-type germlings under the same conditions produced elongated primary and secondary germ tubes with one or more lateral branches (Fig. 1a) and underwent five or more mitotic events after 16 h of incubation.

Complementation and sequencing of wild-type and mutant
alleles. A swoF pyrG strain was transformed with a genomic library constructed in vector pRG3/AMA1, which carries the pyr4 gene and the AMA1 sequence for plasmid autonomous replication (1, 36). A total of 1,092 pyr prototrophs were screened for growth at restrictive temperature (42°C). Three transformants were judged to be complemented based on wild-type colony morphology at the restrictive temperature. These transformants also showed wild-type morphology when examined microscopically (Fig. 1c). Restriction digestion of the transformants showed that the swoF mutant allele contains a change in base 1216 of the genomic sequence from G to T, resulting in a change in protein residue 369 from aspartic acid to tyrosine.

**Protein modeling.** The crystal structures for the C. albicans and S. cerevisiae NMTs have been determined with and without bound substrate peptide and myristoyl-CoA analogues (3, 16, 44). The Nmt1p fold contains a large β sheet bisected by a pseudo-twofold axis and flanked by several α helices. The ligand binding sites are related by a pseudo-twofold axis, with the myristoyl-CoA binding to the N-terminal half of the protein and the peptide substrate binding to the C-terminal half. Using these structures as templates, we generated a homology model for A. nidulans SwoFp (Fig. 5). The N-terminal 76 amino acids are missing from the crystal structures and so are not included in our model. Most of the residues found to be important in myristoyl-CoA binding, peptide binding, or transferase activity are conserved in SwoFp (Fig. 4). Amino acids critical for structural stability are also conserved among all NMTs. The swoF D369Y mutation changes one of these structurally important residues, an aspartic acid that participates in stabilizing a turn in β-strand βk via hydrogen bonds and an ionic interaction (Fig. 5b).

**Partial growth restoration with myristate.** In S. cerevisiae, the addition of myristate restores wild-type growth to most ts nmt mutants (14). Strain AXL19 containing the swoF mutation did not produce a mycelial colony at 42°C (Fig. 6e). However, with the addition of 500 μM myristate, AXL19 produced a filamentous colony (Fig. 6d), although it was smaller than the colony produced by swoF+ strain A773 (Fig. 6b). Microscopically, A773 establishes characteristic long filamentous germ and Fig. 4). The highest identity and similarity (80 and 88%, respectively) were to a predicted NMT from Aspergillus fumigatus found in GenBank. The highest identity and similarity to an experimentally verified NMT were to S. cerevisiae Nmt1p (50 and 65%, respectively). The N-terminal 30 to 100 residues of these proteins were poorly conserved; however, the remaining 350 to 400 residues were highly conserved.

The swoF mutant allele was amplified from AXL19 by PCR. Sequence determination for three replicate PCR clones showed that the swoF mutant allele contains a change in base 350 to 400 residues were highly conserved.
tubes in minimal medium with or without 500 μM myristate (Fig. 6e and h). swoF cells grown in minimal medium at restrictive temperature either failed to establish polarity (Fig. 1d and 6g) or established but could not maintain polarity (Fig. 1b). Viewed microscopically, swoF cells grown in minimal medium amended with 500 μM myristate established and maintained polarity but showed abnormal swelling within spores and hyphae. Germ tubes were also thicker and shorter than those of the wild type in the presence of myristate (Fig. 6f).

**DISCUSSION**

SwoFp is an NMT. The swoF mutant was restored to wild-type growth by introduction of an *A. nidulans* gene predicted to

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**FIG. 4.** Alignment of *A. nidulans* SwoFp (Anid), putative Nmtp from *A. fumigatus* (Afum), Nmt1p from *S. cerevisiae* (Scer), and Nmt1p from *C. albicans* (Calb). Black shading shows residue identity in at least three proteins. Gray shading shows similarity in at least two residues. Secondary structures are shown above the corresponding amino acid sequence as colored arrows (β strands) or cylinders (α helices) coded blue (N terminus) to red (C terminus). The nomenclature for the secondary structures follows that used by Bhatnager et al. (3). Residues involved in binding myristoyl-CoA are indicated by gray stippled bars below the corresponding sequence, residues involved in binding the peptide substrate are indicated by red bars, and residues involved in binding both ligands are indicated by gray and red stippled bars. Tn, sites of transposon insertion disruption at K175 (E8) and at Q415 (B7). Solid black triangles below the sequence indicate residues interacting with D369. *S. cerevisiae* ts mutations reported by Zhang et al. (49) are indicated by a mutant residue enclosed by a black circle below the wild-type residue. The *A. nidulans* swoF mutation is denoted by a mutant residue (Y) enclosed by a red circle below the wild-type D residue.

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**TABLE 1. Identity and similarity of SwoFp and other NMTs**

| Species               | Protein | GenBank accession no. | % Identity | % Similarity | e value |
|-----------------------|---------|-----------------------|-----------|-------------|---------|
| *Aspergillus fumigatus* | Nmtp    | 6505827               | 80        | 88          | 0.0     |
| *Aejellomyces capsulatus* | Nmt1p   | P34763                | 65        | 81          | 0.0     |
| *Saccharomyces cerevisiae* | Nmt1p   | NP 013296             | 50        | 65          | e−114   |
| *Schizosaccharomyces pombe* | Nmt1p   | O43010                | 50        | 65          | e−125   |
| *Candida albicans*     | Nmt1p   | P30418                | 46        | 61          | e−110   |
encode an NMT. Given the high level of sequence conservation among NMTs, the identification of the complementing gene as an NMT gene is unambiguous. The presence of the D369Y mutation in the swoF gene in the mutant argues that the NMT is not a suppressor of swoF. Myristate amendment of medium restores wild-type growth to nmt mutants in S. cerevisiae, Cryptococcus neoformans, and other organisms (23, 30). Indeed, the first S. cerevisiae nmt mutant was discovered as a myristate auxotroph (33). Thus, the ability of myristate to partially restore wild-type growth to the swoF mutant further argues that the mutant lesion is within the NMT gene. Myristate remediation is thought to increase the pools of myristoyl-

FIG. 5. Ribbon representation of the A. nidulans SwoFp homology model. (a) The SwoFp homology model lacking the first 76 residues is portrayed in a ribbon representation with secondary structure features colored blue (N terminus) through red (C terminus). The substrate molecules are shown in ball-stick representation with myristoyl-CoA colored gray and a peptide analogue colored red. The side chain of D369 projects from β-strand βk and is shown in ball-stick representation and colored black. Helix αH is at the lower right of this view and is colored yellow. (b) Enlargement of the region near D369. The view is rotated about 90° relative to that in panel a, looking down the arrow toward D369. The side chains of T368, D369, T399, and R413 are shown in ball-stick representation. Broken lines represent hydrogen bonds. Bond distances are indicated.
CoA enough to overcome any myristoyl-CoA binding defect found in mutant Nmt proteins. It is also possible that myristate stabilizes the folding of the mutant protein. In this light, it is interesting that myristic acid allows only limited growth of the swoF mutant. Perhaps lower levels of NMT activity are sufficient for the very limited polar growth of yeasts such as S. cerevisiae and C. neoformans but are not sufficient for the highly polar growth of filamentous fungi such as A. nidulans.

Many NMT orthologues have been reported in the literature, including representatives from S. cerevisiae (15), C. albicans (46), A. fumigatus (30), H. sapiens (17). In addition, NMT orthologues from Schizosaccharomyces pombe and A. fumigatus are present in GenBank. NMT genes are single-copy genes in all fungi in which they have been characterized (30). Disruption of this single copy is lethal in S. cerevisiae (15), C. albicans (43), and C. neoformans (30). NMTs from C. albicans, C. neoformans, and H. sapiens functionally complement the lethal S. cerevisiae null allele (31). A preliminary search of the A. nidulans expressed sequence tag database (http://www.genome.ou.edu/asperblast.html) indicates only one NMT in A. nidulans. Additionally, in Southern analysis of A. nidulans genomic DNA, a swoF probe hybridizes to only one band (B. D. Shaw and M. Momany, unpublished data).

SwoFp homology model. Using the X-ray crystal structures of NMTs from S. cerevisiae and C. albicans as templates, we homology modeled A. nidulans SwoFp (Fig. 5). Because most residues involved in substrate binding, catalysis, and structural stability among the NMTs are absolutely conserved, our model is likely very accurate. As can be seen in the template structures, the predicted SwoFp consists of two β sheets related by a pseudo-twofold symmetry axis and flanked by α helices. Most of the ts mutations previously reported for NMTs are either in the central core or in residues very near the substrate binding sites and result in higher km values for peptide binding and on the face opposite those for the wild type (Fig. 4) (3, 49). We expected that the D369Y lesion in the swoF mutant would also fall within the core or near the pockets where myristoyl-CoA and peptide ligands bind. However, the mutated residue is at the amino-terminal end of β-strand βk, near the surface of the protein and on the face opposite that of the substrate binding sites. The carboxy-terminal end of β-strand βk interacts with the peptide substrate. Therefore, one explanation for the temperature sensitivity is that the D369Y mutation distorts the orientation of strand βk and thus distorts peptide substrate binding. It seems more likely, however, that the D369Y mutation destabilizes the tertiary structure in the local region around strand βk and thus has a more global effect. Figure 5b illustrates the local environment around D369. In the wild-type protein, D369 stabilizes a bend at the amino-terminal end of βk by hydrogen bonding to T368 and bridges the side chains of neighboring secondary structures via an ionic interaction with K413 (helix αH) and hydrogen bonds with T368 and T399 (β-strand βi). T368 and D369 are absolutely conserved across species (49), suggesting that these residues play a critical role in NMTs. Although K413 and T399 are not conserved in higher eukaryotes, conservative replacements, such as K for R413 and S for T399, could maintain these interactions and substitute functionally. While modeling suggests that the bulky side chain of Y369 in the swoF mutant could be accommodated with some adjustment of the surrounding residues, the stabilizing effects of the ionic and hydrogen bonds would be completely lost. Destabilization of strand βk in the mutant could result in lowering SwoFp activity levels through conformational changes affecting substrate binding and catalytic function. Alternatively, destabilization might lead to lower steady-state levels of SwoFp either from improper folding or from increased proteolytic sensitivity. If improper folding is the main defect in SwoFp, it raises the intriguing possibility that myristoylation reme diation acts by furnishing a scaffold around which the myristoyl-CoA binding pocket can be folded or stabilized.

Conclusion. Polar growth is essential for the pathogenicity of A. fumigatus and many other fungal pathogens of animals and plants. Polar growth is a two-step process, and SwoFp plays a role in the first step (polarity establishment) and is absolutely required for the second step (polarity maintenance). Our current model for the involvement of SwoFp in polarity is that a target protein(s) required for polar growth must be myristoylated to be properly localized and/or fully active. At restrictive temperature, mutant SwoFp is unable to properly myristoylate its target. In this light, a recent report that an sgdD germination mutant is defective in a malonyl-CoA synthetase (36) is intriguing. This protein is involved in the formation of acetyl-CoA, a necessary precursor in the production of myristoyl-CoA. It is possible that sgdD is needed for the production of the myristoyl-CoA necessary for the myristoylation of an as-yet-unknown target responsible for the initiation of germination and polar growth.

NMTs are a highly conserved class of eukaryotic proteins; however, their substrate specificities have diverged, with fungal NMTs generally having targets different from those of mammalian NMTs (12, 13, 27, 30, 38). This target divergence, coupled with the fact that deletion of the single copy of the NMT gene in fungi is lethal (15, 30, 43), has led to considerable interest in NMTs as possible targets for a new class of antifun-
agal drugs. This approach has met with success in C. albicans and C. neoformans, for which compounds that specifically inhibit myristoylation are being tested (11, 12, 29, 42). To our knowledge, this option has not yet been explored for A. fumigatus, now the most common fungal pathogen found in autopsies of immunocompromised patients in European hospitals (19). This antifungal potential, coupled with the key role that myristoylation plays in polar growth (a critical aspect of fungal pathogenesis), is a fruitful area for further investigation.

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