Dsc Orthologs Are Required for Hypoxia Adaptation, Triazole Drug Responses, and Fungal Virulence in Aspergillus fumigatus

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Hypoxia is an environmental stress encountered by Aspergillus fumigatus during invasive pulmonary aspergillosis (IPA). The ability of this mold to adapt to hypoxia is important for fungal virulence and genetically regulated in part by the sterol regulatory element binding protein (SREBP) SrbA. SrbA is required for fungal growth in the murine lung and to ultimately cause lethal disease in murine models of IPA. Here we identified and partially characterized four genes (dscA, dscB, dscC, and dscD, here referred to as dscA-D) with previously unknown functions in A. fumigatus that are orthologs of the Schizosaccharomyces pombe genes dsc1, dsc2, dsc3, and dsc4 (dsc1-4), which encode a Golgi E3 ligase complex critical for SREBP activation by proteolytic cleavage. A. fumigatus null dscA-D mutants displayed remarkable defects in hypoxic growth and increased susceptibility to triazole antifungal drugs. Consistent with the confirmed role of these genes in S. pombe, both ΔdscA and ΔdscC resulted in reduced cleavage of the SrbA precursor protein in A. fumigatus. Inoculation of corticosteroid immunosuppressed mice with ΔdscA and ΔdscC strains revealed that these genes are critical for A. fumigatus virulence. Reintroduction of SrbA amino acids 1 to 425, encompassing the N terminus DNA binding domain, into the ΔdscA strain was able to partially restore virulence, further supporting a mechanistic link between DscA and SrbA function. Thus, we have shown for the first time the importance of a previously uncharacterized group of genes in A. fumigatus that mediate hypoxia adaptation, fungal virulence, and triazole drug susceptibility and that are likely linked to regulation of SrbA function.

As mortality due to invasive fungal infections (IFIs) continues to remain high despite improved diagnostics and prophylactic use of antifungal drugs, research to better understand fungal pathogenesis mechanisms is important. The aim of this research is to uncover new aspects of fungal physiology or the fungus-host interaction that can be manipulated to improve treatment outcomes. Investigating the host microenvironments encountered by human-pathogenic fungi during the initiation, development, and active stages of subsequent infection and how fungi adapt to these dynamic environments is one area where potential therapeutic opportunities exist.

Recently, there has been an increased interest in how levels of oxygen at the site of infection impact the outcome of IFIs (18, 22). A major reason for this interest is that fungal strains with a deficient ability to adapt to hypoxia fail to cause lethal disease in murine models. Among the best examples of these fungal strains are the Cryptococcus neoformans and Aspergillus fumigatus null mutants of the sterol regulatory element-binding proteins (SREBPs) (Sre1 and SrbA, respectively) (11, 12, 46). SREBPs were first identified in higher eukaryotes as regulators of cholesterol and lipid metabolism (7, 19, 20, 37, 45). Mammalian SREBPs are synthesized as endoplasmic reticulum (ER) membrane-bound precursors and contain two transmembrane domains separated by a short luminal loop. The C terminus contains a domain that binds to the SREBP cleavage-activating protein (SCAP), which senses sterol content within the cell. Under low sterol levels, transport of SREBP to the Golgi apparatus is triggered, where SREBP undergoes two sequential proteolytic cleavage events mediated by site 1 (S1P) and site 2 (S2P) proteases. The first cleavage event takes place in the luminal loop, followed by a second cleavage event that separates the N terminus from the transmembrane domain. These cleavage events allow the N terminus, containing a basic helix-loop-helix (bHLH) transcription factor, to translocate to the nucleus and regulate gene transcription (8–10).

In fungi, an SREBP ortholog (Sre1) was first identified and characterized in the fission yeast Schizosaccharomyces pombe, where Sre1 in combination with an SREBP cleavage-activating protein (SCAP) detects intracellular sterol levels as an indirect sensor of oxygen (24, 44). Sre1 activation in S. pombe is regulated in part by proteolytic cleavage, as are SREBPs in humans and insects (8, 24, 37). In C. neoformans, Sre1 is also proteolytically cleaved in response to hypoxia, and this cleavage involves a site 2 protease, as found in mammals (2). Recently, cleavage of Sre1 in S. pombe was found to be dependent on a Golgi E3 ligase complex encoded by dsc genes and not site 1 and 2 proteases, which S. pombe appears to lack (42, 43). This result illustrates that while many aspects of SREBP regulation are conserved across organisms, major differences exist. For example, in the model invertebrate Drosophila melanogaster, alternative mechanisms of SREBP cleavage have also been reported (1, 37).

Mechanisms of SREBP regulation in the pathogenic mold A. fumigatus are currently unknown. Bioinformatic analyses in A.
fumigatus predict that SrbA has two transmembrane domains, suggesting that as with SREBPs in other organisms, SrbA is an integral membrane protein that needs processing to release the N terminus containing the bHLH DNA binding domain. However, as in S. pombe, neither an S1P nor an S2P that could be responsible for proteolytic cleavage has been found in A. fumigatus. Moreover, in contrast to S. pombe, there is no identifiable SCAP ortholog in A. fumigatus, at least through sequence-based bioinformatic analyses (8, 12, 24, 47). The absence of SCAP in A. fumigatus raises the question how, or whether, SrbA in A. fumigatus is involved in sterol sensing. The potential absence of SCAP, S1P, and S2P in A. fumigatus may also suggest that regulated intramembrane proteolysis is not an important regulation mechanism for SrbA activation in A. fumigatus.

Here we present data that suggest regulation of SrbA activity in A. fumigatus occurs in part through a group of previously uncharacterized genes in Aspergillus spp. that are orthologs of the S. pombe Dsc E3 ligase Golgi complex-encoding genes that are critical for Sre1 processing in fission yeast. In A. fumigatus, we find that the major phenotypes of the SrbA null mutant, including loss of fungal virulence and responses to triazole antifungal drugs, are largely phenocopied in the Dsc null mutants. Processing of the SREBP SrbA in A. fumigatus also appears to be altered in the absence of DscA and DscC, and reconstitution (rec) of DscA with the N terminus of SrbA can partially restore wild-type phenotypes. Taken together, our data show an important role in fungal virulence and triazole drug responses for the dsc genes in A. fumigatus that are likely in regulation of SrbA function through an undefined mechanism.

MATERIALS AND METHODS

Fungal strains, media, and growth conditions. All strains used in this study are listed in Table 1. Conidia were stored as frozen stocks with 50% glycerol at −80°C. The strains were routinely grown in glucose minimal medium (GMM) with appropriate supplements at 37°C as previously described (40). To prepare solid media, 1.5% agar was added before medium (GMM) with appropriate supplements at 37°C as previously described (14).

TABLE 1 Fungal strains used in this study

| Strain     | Description                  | Reference |
|------------|------------------------------|-----------|
| CBS 144.89 | Wild type                    | From J. P. Latgé, Institut Pasteur |
| CEA17      | Wild type with pyrG−         | 16, 17    |
| SDV1       | srbA::pyrG                   | 44        |
| TDC14.9    | gpdA(p):SrbA::GFP           | This study |
| TDC19.77   | srbA(p):GFP::SrbA            | This study |
| TDC8.26    | srbA::srbA aa1-425::GFP      | This study |
| TSW27.8    | dscA::pyrG + srbA aa1-425   | This study |
| NSSbA      | srbA::pyrG + srbA aa1-425   | This study |
| ∆dscA      | dscA::pyrG                   | This study |
| ∆dscB      | dscB::pyrG                   | This study |
| ∆dscC      | dscC::pyrG                   | This study |
| ∆dscD      | dscD::pyrG                   | This study |
| dscA rec   | dscA::pyrG + dscA            | This study |
| dscB rec   | dscB::pyrG + dscB            | This study |
| dscC rec   | dscC::pyrG + dscC            | This study |
| dscD rec   | dscD::pyrG + dscD            | This study |

Strain construction. The A. fumigatus orthologs (dscA, dscB, dscC, and dscD, here referred to as dscA-D) of the S. pombe genes dsc1, dsc2, dsc3, and dsc4 (dsc4-1) identified were as follows: dsc1, AFU8_011550 or AFUA_G12080; dsc2, AFUB_068690 or AFUA_4G11680; dsc3, AFUB_067780 or AFUA_4G10700; and dsc4 AFUB_040760 or AFUA_3G08340. Generation of dscA-D deletion mutants in A. fumigatus strain CEA17 was accomplished by replacing the open reading frame of target genes with A. parasiticus pyrG. To construct the gene replacement cassette, a three-piece overlap PCR strategy was used as previously described (15). The gene replacement cassettes were generated by amplifying two approximately 1.0-kb sequences homologous to the genes of interest using primers for the 5′ sequence and the 3′ sequence (primer sequences available upon request). The approximately 3.0-kb sequence of the A. parasiticus pyrG gene was amplified from plasmid pJW24 (gift of Nancy Keller, University of Wisconsin-Madison). All three PCR products were combined in equimolar concentrations and served as the template in a final PCR. Nested primers were used to amplify a fragment that was then cloned into the vector pDONR221 through the BP reaction (Invitrogen). The resulting plasmids were used as the template to amplify the gene replacement cassettes for use in fungal transformation.

To generate strains carrying the N terminus of srbA, the DNA sequence for the first 425 amino acids (aa), including −1,200 bp of the promoter region, were inserted into the plasmid pBC-hygro (41) (Fungal Genetics Stock Center [30, 31]), which was linearized with EcoRI and Spel. The resulting plasmid, pSrbA_N-term-native promoter, was used as a template to amplify the whole construct encoding the first 425 aa of SrbA (−6.7 kb) including the hygromycin B resistance marker in front of the SrbA construct for use in fungal transformation. To express NSrbA in ∆dscA, the same construct that was used to complement ∆SrbA by NSrbA was used. The final 6.7-kb PCR product was amplified by primers pBC-hygro-fw and pBC-hygro-rev and transformed into ∆dscA.

Green fluorescent protein (GFP)-tagged SrbA constructs were made for localization studies. To generate a construct to express SrbA::GFP (C-tag) using an A. nidulans gpdA promoter, a 1.9-kb gpdA(p) fragment was PCR amplified from A. nidulans wild-type DNA using primers gpdA-SrbA-gfp1 and gpdA-srbA-gfp2. A 3.0-kb srbA coding sequence was amplified from A. fumigatus wild-type DNA using primers gpdA-SrbA-gfp3 and SrbA-GFP-Fu1. A 2.7-kb gfp fragment including a GA5 linker, gfp coding sequence, and A. fumigatus pyrG was amplified from plasmid pFN03 (obtained from the Fungal Genetics Stock Center) using primers pFN03F and pFN03R. A 7.4-kb final construct was generated via a double-joint PCR method using primers gpdA-SrbA-gfpNewF and pFN03R and transformed into A. fumigatus wild-type CEA17.

To generate a GFP:SrbA (N-tag) expression construct, three DNA fragments were PCR amplified and linked together via a double-joint PCR method. A 1.4-kb srbA native promoter fragment was amplified using primers GFP-SrbA1 and GFP-SrbA2 from A. fumigatus wild-type genomic DNA, a 760-bp gfp fragment including gfp coding sequence and a GA5 linker was amplified using primers GFP-SrbAne1 and gpdA-gfpSrbA7 from plasmid pFN03, and a 3.6-kb srbA fragment including an srbA coding sequence and a 550-bp downstream sequence of srbA stop codon was amplified using primers gpdA-gfpSrbA8 and GFP-SrbA6 from A. fumigatus wild-type genomic DNA. A 5.5-kb final construct was amplified using primers GFP-SrbAnewF and GFP-SrbAneR and cotransformed with pJW24 into A. fumigatus CEA17. This generated the GFP: SrbA (N-tag) strain.

Generation of fungal protoplasts and polyethylene glycol-mediated transformation of A. fumigatus were performed as previously described (6). Homologous recombination and gene replacement were confirmed by Southern blot analysis with the digoxigenin labeling system (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (14).

Quantitative real-time PCR. A. fumigatus strains were cultured in liquid GMM under normoxia or hypoxia. Germinals and mycelia were collected with vacuum filtration and lyophilized with a freeze dryer (Labconco) prior to homogenization with 0.1-mm glass beads. Total RNA was extracted using the TRIzol reagent (BioLine) according to the manufacturer’s instructions.

The resulting RNA was then treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer’s instructions. Total RNA concentrations were measured at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Two micrograms of RNA were then reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen) down stream sequence of srbA stop codon was amplified using primers gpdA-gfpSrbA8 and GFP-SrbA6 from A. fumigatus wild-type genomic DNA. A 5.5-kb final construct was amplified using primers GFP-SrbAnewF and GFP-SrbAneR and cotransformed with pJW24 into A. fumigatus CEA17. This generated the GFP: SrbA (N-tag) strain.

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turer's protocol and subsequently further purified with the Qiagen RNeasy plant minikit (Qiagen). Genomic DNA contamination was fur-
ther removed with Turbo DNase I treatment (Ambion). DNase-treated total RNA (500 ng) was reverse transcribed with the Quantitect reverse transcriptase kit (Qiagen), quantitative real-time PCR (qPCR) was subse-
quently performed, as we have previously described [13]. A no-DNA template control was used in each analysis. Each sample was tested in triplicate, and data were normalized to the tef α reference gene and are presented relative to the normalized expression of genes in the wild-type control (29).

Plate assays. Colony growth was quantified as previously described (32).

Hyphotic cultivation. Normoxic conditions were considered general
aspheric conditions within the lab (~21% O2). For hyphotic conditions, a
hyphotic incubation chamber (Invivo; 400; Ruskin) was used. The cham-
ber was maintained at 37°C and kept at 1% O2, 5% CO2, and 94% N2
controlled through a gas mixer (Gas Mixer Q; Ruskin/Baker Company).

Protein extraction and assay. For protein extraction, conidia of the
various strains were grown in 60 ml of liquid GMM in baffle flasks at 23°C
and shaken at 250 rpm for 17 h to generate germlings. Fungal cultures
were then grown from germlings for 30 min, 1 h, and 2 h at 37°C under
normoxic or hypoxic conditions. Fungal cultures were filtered using
Whatman no. 54 filter paper on a Buchner funnel with gentle suction. The
dried tissue was powdered in liquid nitrogen, and lyophilized for 8 h. The
dried tissue was powdered in liquid nitrogen, and 60 to 80 mg was extracted
using the equivalent of 1 ml per 100 mg of a urea- and detergent-contain-
ing buffer (33) with additional detergent (Tigel) at a final concentration of
0.1% and 10 μl/ml each of HALT protease inhibitors (Thermo Scientific)
and 100 mM PMSF. The powdered tissue was vortexed with extraction
buffer at room temperature. Extracted samples were centrifuged for 5 min
at 16,000 × g, and the supernatant was collected and diluted 1 part with
0.4 parts (vol/vol) of a solution equivalent to the extraction buffer but not
containing urea or detergents, to reduce viscosity. A further 10-fold dilu-
tion in sterile water was used for the Pierce 660-nm protein assay in the
presence of Ionic detergent compatibility reagent (Thermo Scientific)
with bovine serum albumin (BSA) as a standard.

Immunoblots. Ten-well 10% Mini-Protein precast gels (Bio-Rad)
were used for SDS-PAGE. Denatured protein was loaded (40 μg per well).
After gel electrophoresis, protein was transferred to a nitrocellulose mem-
brane (Hybond-C Extra; Amersham Biosciences). Srbα was detected on
blots using the Srbα1–275 recombinant primary N terminus antibody at a
1/27,000 dilution and an anti-rabbit alkaline phosphatase (AP)-conju-
gated secondary antibody raised in goat (Abcam) at a 1/5,000 dilution.
Chemiluminescence was measured following incubation of blots with
Tropix CPD Star substrate (Applied Biosystems) with Immun-star en-
hancer (Bio-Rad) using a FluorChem FC2 image (Alpha Innotech). For
loading controls, anti-γ-tubulin (Sigma, T5192) (human) was utilized.

Murine virulence test and histopathology. CD1 female mice, 6 to 8
weeks old, were obtained from Charles River Laboratories (Raleigh, NC)
or from a breeding colony located in the Animal Resources Center (ARC)
at Montana State University. Mice were housed five per cage in an envi-
ronment of HEPA-filtered air, with access to autoclaved food and sterile
acidified water ad libitum. Mice were immunosuppressed with a single
40 mg/kg dose of triamcinolone (Kenalog; Bristol-Myers Squibb Com-
pany, Princeton, NJ) injected subcutaneously 1 day prior to infection with
A. fumigatus conidia. For survival studies, 10 or 20 mice per A. fumigatus
strain were used, and for histology, 5 mice per strain were used. Animals
were inoculated intranasally with 2 × 106 conidia in 40 μl of 0.01% Tween
and monitored twice a day for 14 days. Histology was carried out on day 3
postinoculation. No mock-infected animals died using this protocol. An-
imal experiments were repeated in duplicate. For histology, lung tissue
was fixed in 10% phosphate-buffered formalin, embedded in paraffin,
ceptibility to the triazoles fluconazole and voriconazole (5, 47). We have previously found that loss of SrbA results in increased susceptibility to other triazole drug responses to that of the wild type.

The dsc genes are required for virulence in A. fumigatus. Unlike the wild type, the Dsc orthologs in S. pombe Dsc3 is most similar to A. fumigatus DscC. DscC contains 2 transmembrane domains and 2 conserved domains of unknown function in the N and C termini of the protein, respectively. Finally, S. pombe Dsc4 is most similar to A. fumigatus DscD. DscD contains 3 transmembrane domains and a conserved domain of unknown function that is conserved in fungi. Taken together, in silico sequence analyses strongly suggest that A. fumigatus DscA-D represent potential orthologs of S. pombe dsc1-4.

Dsc proteins are required for growth during hypoxia and triazole drug susceptibility. In order to determine the function of the A. fumigatus Dsc proteins, we made null mutants and reconstituted strains of dscA-D. Null mutants and reconstituted strains were confirmed with Southern blot and PCR analyses (see Fig. S1 in the supplemental material). Similar to the ΔsrbA strain, ΔdscA, ΔdscB, and ΔdscD strains display a remarkable inability to grow on solid media under hypoxia (Fig. 1). Under normoxia, all 4 Dsc null mutants generally display wild-type growth rates and colony morphology. In contrast, growth of Δdsc mutants generally display wild-type growth rates and colony morphology. In contrast, growth of Δdsc mutants generally display wild-type growth rates and colony morphology.

Phenotype similarities between the dsc mutants and the ΔsrbA strain were also observed in tests for triazole susceptibility. We have previously found that loss of SrbA results in increased susceptibility to the triazoles fluconazole and voriconazole (5, 47). The ΔdscA, ΔdscB, and ΔdscD strains, like the ΔsrbA strain, also displayed significant increases in fluconazole and voriconazole susceptibility as measured with Etest (Fig. 2A and B). However, the ΔdscC strain was less susceptible to both triazoles as well as other dsc mutants (Fig. 2A and B). For each of the dsc mutants, ectopic reconstitution of the wild-type allele restored antifungal drug responses to that of the wild type.

FIG 1 Growth defect of dsc mutant strains under hypoxia. Radial growth of ΔdscA-D, wild-type (CBS 144.89) and ΔsrbA cells after inoculation of GMM plates with 10^5 conidia and incubation at 37°C for 96 h under normoxia and hypoxia (1% O2 and 5% CO2). ΔsrbA, ΔsrbA deletion strain. Unlike the other dsc deletion strains, the ΔdscC strain showed a small amount of growth under hypoxia but much less than the wild type. Full or partial restoration of growth of the dsc mutants under hypoxia was achieved by reconstitution (rec) with the native allele.

Der1 family of proteins, which are involved in ER-associated degradation (ERAD) and have previously been implicated in the regulation of the ER stress response (47). Since we showed that the loss of dsc gene function results in in vitro phenotypes similar to the loss of srbA, we tested the virulence of ΔdscA, ΔdscC, ΔsrbA, and associated reconstituted strains. Given that the ΔdscB and ΔdscD strains were phenotypically identical to the ΔdscA strain, we elected not to test these strains in our animal model. We used an IPA model that utilizes the synthetic corticosteroid triamcinolone acetonide (Kenalog) for immunosuppression. This model is characterized by the development of extensive pulmonary hypoxia at sites of A. fumigatus infection (21). As observed in our two previous murine models of IPA, the ΔsrbA strain was severely attenuated in virulence in this corticosteroid model (P < 0.001) (Fig. 3A and B). Loss of Δdsc also resulted in a significant virulence attenuation as measured by murine survival (P < 0.001) (Fig. 3A). Despite a moderate amount of growth under hypoxia, the ΔdscC strain was also highly attenuated in virulence (P < 0.001) (Fig. 3B). Reconstitution of the respective wild-type alleles in ΔdscA and ΔdscC strains restored virulence comparable to that of the wild type (P = 0.43 and 0.13, respectively). These results suggest that DscA and DscC are required for A. fumigatus to cause lethal disease in a murine model of IPA. To our knowledge, this is the first report of a critical role of these proteins in fungal virulence. Moreover, taken together, these phenotypic data suggest that the loss of Dsc function results in phenotypes that are strikingly similar to those observed upon the loss of SrbA both in vitro and in vivo, suggesting a possible link between Dsc and SrbA functions in A. fumigatus.

Full-length Aspergillus fumigatus SrbA is processed into a smaller form. We next designed a series of experiments to determine the possible relationship between the Dsc proteins and SrbA in A. fumigatus. Based on the reported role of Dsc orthologs in the proteolytic cleavage of the S. pombe SREBPs Sre1 and Sre2 and the apparent absence of SIP and S2P proteases in A. fumigatus, we hypothesized that Dsc protein function was involved in cleavage of SrbA in A. fumigatus. We first sought to determine whether cleavage of full-length SrbA occurs in A. fumigatus as has been observed in other organisms with SREBPs. To answer this question, we utilized a polyclonal antibody directed at detecting amino acids 1 to 275 in SrbA on immunoblots of A. fumigatus protein extracts (4). Protein extracts were generated from A. fumigatus germings grown in GMM liquid cultures at 37°C for 30, 60, and 120 min. Densitometry was used to compare protein bands on immunoblots. The strongest band on immunoblots under normoxia was observed at around 120 kDa, and we hypothesize that this band represents the SrbA precursor protein (P) though it is approximately 10 kDa larger than the protein molecular size predicted by informatics (Fig. 4A). Notably, this band was absent from a protein extract from the ΔsrbA strain and a strain in the...
**ΔsrbA** background expressing SrbA amino acids 1 to 425 (NSrbA) that contains the transcription factor bHLH domain (Table 1). For studying changes in precursor protein levels, densitometry values were normalized to gamma tubulin (Fig. 4B). A band at about ~60 kDa was also found on immunoblots from the wild type, and this was similar in size to the band observed from the NSrbA strain, suggesting that this band is the cleaved N terminus of SrbA containing the bHLH domain. Cleavage was subsequently assessed from the ratio of N-terminal to precursor SrbA densities. As observed in *S. pombe* and *C. neoformans* previously, the abundance of the N terminus transcription factor is increased in response to hypoxia and is most evident at 30 min (Fig. 4C, Hypoxia). Perhaps the most dramatic finding on the immunoblots, and in contrast to results observed in *S. pombe*, was that full-length precursor SrbA abundance dramatically decreased in response to hypoxia in *A. fumigatus* (Fig. 4A). In addition, basal cleavage of SrbA was readily detectable under normoxia (Fig. 4C and data not shown).

Our rationale for constructing NSrbA was in part based on the hypothesis that if processing of full-length SrbA was required for its function as a transcription factor, then reconstitution of ΔsrbA phenotypes would occur in a strain expressing only the N-terminal portion of SrbA that contains the bHLH DNA binding domain. Growth of NSrbA on GMM plates after 96 h under normoxia and hypoxia was compared to that of wild-type and ΔsrbA strains. All strains showed similar growth under normoxia and filled the plate within 96 h (data not shown). Under hypoxia the wild type grew at the same rate as previously observed under normoxia on solid media while the ΔsrbA strain did not grow, as previously reported (Fig. 4D)(47). NSrbA grew under hypoxia but showed a small decrease in growth compared to the wild type (paired t test, *P* = 0.04) (Fig. 4D). qRT-PCR analysis of the truncated transcript revealed that srbA mRNA abundance in NSrbA is ~4-fold higher (data not shown) than in the wild type even though there was only a single ectopic insertion of the DNA construct in this strain. Taking these data together, we conclude that...
their normal functionality (data not shown). Importantly, no GFP signal was detected in the nuclei with SrbA:GFP strains, suggesting that full-length SrbA fused with GFP likely does not enter the nucleus. In contrast, when tagged at the N terminus, the cells expressing GFP:SrbA showed either nuclear localization (Fig. 5C) or ER/NE-localization (Fig. 5D) of GFP signal under the same culture conditions. Cells expressing NSrbA:GFP showed strong nuclear localization signals (Fig. 5E). These observations suggest that precursor SrbA localizes to ER/NE and the cleaved N terminus localizes to nuclei, supporting the hypothesis that SrbA is an integral membrane protein in A. fumigatus that likely requires proteolytic processing for full activity.

**Aspergillus fumigatus Dsc proteins are required for full SrbA cleavage and activation.** We next examined the effects of exposure of the ΔdscA and ΔdscC strains to hypoxia in liquid cultures for 30 min to 2 h to determine whether SrbA cleavage was affected in these A. fumigatus null mutants. At 30 min under normoxia, no marked detectable difference in SrbA precursor or cleavage could be detected in ΔdscA and ΔdscC strains compared to wild-type samples. However, at 30 min under hypoxia, a marked difference in both SrbA precursor abundance and cleavage was observed in ΔdscA and ΔdscC strains compared to wild-type samples (Fig. 6A to C). In ΔdscA and ΔdscC samples, the dramatic decrease in SrbA precursor in response to hypoxia was not observed and a substantial, but not complete, loss in cleavage was observed. After 1 or 2 h under hypoxia, there was partial loss of cleavage in ΔdscA and ΔdscC strains compared to wild-type samples, and at these time points the loss of precursor protein also started to become apparent.

To further confirm the immunoblotting and phenotype results with potential activation of SrbA function, we next examined the mRNA abundance of 3 known SrbA target genes in the ΔdscA and ΔdscC strains. Recently, we confirmed that SrbA is a direct regulator of erg11A (cyp51A), erg25A, and srbA mRNA abundance via binding to the respective promoter sequences directly (4, 5). We examined erg11A, erg25A, and srbA mRNA abundance in the ΔdscA and ΔdscC strains after exposure to hypoxia for 1 h. ΔsrbA and ΔdscA strains had negligible amounts of mRNA for the two erg genes, while the ΔdscC strain also had reduced (but greater than those of the ΔdscA strain) amounts of mRNA abundance for erg11A and erg25A (Fig. 6D). With regard to srbA mRNA, it was virtually undetectable as expected in the ΔsrbA strain at 1 h after exposure to hypoxia. srbA mRNA was markedly reduced in the ΔdscA strain but surprisingly not statistically different in the ΔdscC strain compared to the wild type. Taken together, these data strongly suggest that the loss of dsc genes in A. fumigatus alters SrbA processing and subsequent SrbA target gene expression, further supporting a link between the Dsc null mutant phenotypes and loss of SrbA function.

**Virulence of the ΔdscA strain can be partially restored via ectopic expression of NSrbA.** To determine if the dsc mutant virulence phenotypes were due to loss of SrbA function, we carried out survival tests with the ΔdscA strain reconstituted with NSrbA using the corticosteroid murine model (Fig. 7A). We also separately but concurrently performed histopathological analyses of lungs from infected mice in order to observe fungal growth and host responses 3 days postinfection (Fig. 7B). As a control we tested survival of the ΔsrbA strain reconstituted with NSrbA and network (ER/nuclear envelope (NE) (Fig. 5B).
found that it was as virulent as the wild type (Fig. 7A) ($P = 0.88$). NSrbA thus was an effective replacement for endogenous SrbA in this strain. As shown previously (Fig. 3A), the $\Delta srbA$ strain was avirulent in this murine model, and inoculation with this strain did not produce an effect different from that of mock injection ($P = 0.48$). Typically, in lung sections stained with Gomori’s methenamine silver (GMS) from mice inoculated with the $\Delta srbA$ strain, only short germlings were found, and hematoxylin and eosin (H&E) staining showed inflammatory cells in infected areas recruited to clear the infection (Fig. 7B). Reconstitution of the $\Delta srbA$ strain with NSrbA increased virulence compared to that of the $\Delta srbA$ strain ($P = 0.005$) but not to the level of the wild type ($P < 0.001$). Histology showed that reconstitution of the $\Delta srbA$ strain with NSrbA increased growth of the fungus and colonization in the lungs as abundant elongated hyphae were visible in multiple lesions. There was an associated influx of undefined inflammatory cells into the lungs, as was also observed for the $\Delta srbA$ strain reconstituted with NSrbA and wild-type cells (Fig. 7B). Taken together, these data suggest that the loss of DscA function in A. fumigatus can partially be compensated for in a murine model of IPA by ectopic introduction of NSrbA. These results further support a potential link between DscA and SrbA function in A. fumigatus.

**DISCUSSION**

The SREBP SrbA in A. fumigatus has previously been shown to be required for hypoxia and low-iron adaptation, triazole drug susceptibility, and virulence (4, 5, 47). Given the potential importance of SrbA in fungal virulence and responses to antifungal therapy, a further examination of the mechanisms of SrbA regulation and function is warranted in A. fumigatus. SREBPs exist as precursor proteins that are cleaved to release the active N terminus transcription factor (8). In A. fumigatus, whether SrbA required proteolytic processing for function as a transcription factor was unclear. Bioinformatic analyses of the SrbA amino acid sequence strongly suggest that SrbA is an integral membrane protein like SREBPs in other organisms. Here, we observed that the GFP signal from an SrbA:GFP fusion protein strain largely localizes to the nuclear envelope/ER membrane, supporting the bioinformatics data with regard to SrbA localization in A. fumigatus. However, previous attempts to identify the sterol cleavage activating protein (SCAP) in A. fumigatus that serves as the sterol sensor and important regulator of SREBP processing in other organisms have been unsuccessful. In addition, no clear homologs of the S1P and S2P that are required to release active SREBP from the membrane in some organisms are identifiable in A. fumigatus by sequence-based methods. Taken together, these observations hint at possible distinct mechanisms of SREBP regulation in the mold A. fumigatus and raise the question whether regulated intramembrane proteolysis is important for SrbA activity in this filamentous fungus.

Similar to A. fumigatus, the fission yeast S. pombe also appears to lack S1P and S2P. Recently, utilizing a forward genetics approach, Stewart and colleagues identified a group of genes ($dsc1-4$)
encoding a transmembrane Golgi E3 ligase complex required for fission yeast SREBP cleavage (43). Named the Dsc (defective for SREBP cleavage) complex, it binds SREBP and requires components of the ubiquitin proteasome pathway to induce Sre1 and Sre2 cleavage. This result had precedence, as an important role of the proteasome is to carry out limited proteolysis of certain ubiquitin-modified precursor transcription factor proteins (36). For example, this regulated ubiquitin/proteasome-dependent processing (RUP) of mammalian p105 produces the active p50 subunit of the transcription factor NF-κB (28, 34). In yeast, two related inactive precursor proteins, SPT23 and MGA2 (yeast homologs of NF-κB), located in the ER are also processed by RUP to produce the transcription factor p90 (23). In addition to dsc1-4, Stewart et al. recently reported that Dsc5 and Cdc48 are additional components required for fission yeast Sre1 cleavage (42). As A. fumigatus appears to lack S1P and S2P, here we tested the hypothesis that a similar SREBP cleavage mechanism may exist in this human-pathogenic mold.

Bioinformatic analyses of the A. fumigatus genome sequence using the S. pombe Dsc1-4 amino acid sequences revealed that A. fumigatus contained putative uncharacterized orthologs of these key SREBP-associated proteins. While A. fumigatus also appears to contain homologs of Dsc5 (AFUB_013870) and Cdc48 (AFUB_032770) (data not shown), here we report on our initial characterization of the putative Dsc1-4 orthologs. Phenotypic analyses of the dscA-D null mutants in A. fumigatus revealed a remarkable similarity with our previously reported phenotypic analyses of the SrbA null mutant. In general, DscA, DscB, and DscD null mutants phenocopied each other and the SrbA null mutant with regard to growth under hypoxia and responses to triazole antifungal drugs. In contrast, the DscC null mutant displayed a marked reduction in, but not complete loss of, growth under hypoxia and only a small increase in susceptibility to the triazole drugs. However, despite these in vitro phenotypic differences, the DscC null mutant also displayed a severe reduction in virulence.

A major question resulting from these phenotypic analyses is that of the relationship between these Dsc proteins and SREBP function in A. fumigatus. Our data support the conclusion that the A. fumigatus Dsc proteins, as in S. pombe, play an important role in mediating SrbA activity through an undefined mechanism. In addition to the similar phenotypes observed in the absence of the Dsc proteins in A. fumigatus compared to the ΔsrbA strain, the loss of
DscA and DscC reduced (but did not fully eliminate) SrbA precursor cleavage under normoxic and hypoxic conditions as observed via immunoblot analyses. Moreover, the reconstitution of ΔSrbA and ΔDscA phenotypes with the NSrbA construct supports the conclusion that the observed SrbA precursor cleavage results in a functional transcription factor, though this remains to be definitively determined. In partial support of the conclusion that dsc genes are required to produce active SrbA transcription factor, mRNA abundance of the known SrbA target genes, srbA, erg11A (cyp51A), and erg25A, was markedly reduced in the ΔdscA strain and to a lesser extent in the ΔdscC strain, suggesting that SrbA activity was attenuated in these null mutants. Intriguingly, the mRNA abundance data of known SrbA target genes correlated with the magnitude of the observed phenotypic responses of the ΔdscA and ΔdscC strains to hypoxia and triazole drugs. Moreover, the mRNA abundance data also support the conclusions drawn from the immunoblotting experiments that low levels of SrbA cleavage still occur in ΔdscA and ΔdscC strains. How these Dsc proteins mediate SrbA function in *A. fumigatus* remains largely unclear from our studies presented here and will be the subject of future investigation.

With regard to SrbA regulation in *A. fumigatus*, our data not surprisingly suggest that, as in other organisms, cleavage of full-length SREBP also occurs in this filamentous fungus. However, our immunoblot assays suggest two potentially novel features of SREBP regulation in *A. fumigatus* that await further study. First a key observation from our immunoblot analyses is the observation that cleavage of SrbA in *A. fumigatus* was essentially detectable under all conditions tested, including under normoxic conditions. We cannot definitively rule out that full-length precursor SrbA is an unstable protein in *A. fumigatus* and possibly susceptible to *in vitro* proteolysis during protein extraction. Evidence against this alternative conclusion comes from previous observations that SrbA is also required for normal growth under normoxia as exhibited by the approximate 50% reduction in biomass and hyphal morphology defects observed in ΔsrbA under normoxic liquid cultures. We also currently cannot definitely rule out that filamentous fungal growth in shake flask cultures results in low levels of hypoxia in the interior of the fungal microcolonies that tend to form during this form of cultivation. This finding is worthy of further study, as it could suggest that shake flask cultures of filamentous fungi contain mixtures of cells exposed to normoxic and hypoxic conditions, which has important implications for gene expression and other phenotypic analyses with these organisms.

These results, in combination with the previous observations that essential components of the regulated intramembrane proteolysis mechanism appear to be absent in *A. fumigatus*, thus bring into question the mechanisms of SrbA regulation in this pathogenic mold. While our results do not rule out potential regulation at the level of proteolysis, they may suggest that a major aspect of SREBP regulation in *A. fumigatus* occurs via regulating the stability of the N-terminus transcription factor. This is a particularly attractive hypothesis given the apparent absence of SCAP in *A. fumigatus* and raises the question of how proteolysis is regulated in

![Figure 6](https://example.com/figure6.png)

**FIG 6** Reduced hypoxia-induced precursor SrbA cleavage and erg11A and erg25A transcript abundance in *dsc* mutants. (A to C) Immunoblots using protein extracts from cultures of *A. fumigatus* ΔdscA (A), ΔdscC (B), and wild-type (CBS 144.89) (C) cells. Germlings (time zero) were grown in baffle flasks for 30 min, 1 h, and 2 h at 23°C under normoxia (N) or hypoxia (1% O₂ and 5% CO₂) (H). Germlings were grown from conidia at 23°C for 17 h. On the right of the blot, P indicates the precursor SrbA protein band and N indicates the N terminus protein band. NSrbA is a protein extract from a strain expressing the first 425 amino acids of full-length SrbA. ΔsrbA is a srbA deletion strain. Gamma tubulin present in extracts is shown below the main blot. (D) Comparison of transcript abundance normalized to the *A. fumigatus* tefA strain for mycelia from wild-type (CBS 144.89), ΔsrbA, ΔdscA, and ΔdscC strains grown from germlings (time zero) in liquid GMM with agitation for 1 h under hypoxia (1% oxygen and 5% CO₂). Germlings were grown from conidia at 23°C for 17 h. Total RNA was extracted and treated with DNase. Equal amounts of DNase-treated RNA (500 ng) were reverse transcribed and used for qRT-PCR. Error bars represent the standard deviations of the means for 3 biological replicates.
fumigatus is the dramatic loss of SrbA precursor protein observed via immunoblot analyses under hypoxic conditions. S. pombe responds to anaerobic conditions with a gradual increase in SREBP precursor protein levels that correlate with sre1+ mRNA levels, while exposure of C. neoformans to 1% or 0.2% oxygen also causes an increase in SREBP precursor protein (11, 24). In S. pombe, it has been observed that positive feedback regulation at the sre1+ promoter increases Sre1 precursor synthesis that is required for maximal Sre1 activation (26). We previously observed positive transcriptional control of srbA via binding of SrbA to the srbA promoter and a general increase in SrbA mRNA abundance in response to hypoxia that is consistent with this yeast model (4). Thus, the loss of SrbA precursor in A. fumigatus in response to hypoxia was surprising. A potentially intriguing hint at the mechanisms in play in A. fumigatus may come from the observation that SCAP null mutants in S. pombe display rapid degradation of Sre1 precursor by the proteasome and the ER-associated degradation (ERAD) components Ubc7 and Hrd1 (26). Thus, while SrbA in A. fumigatus may be critical for autoregulation of srbA mRNA abundance as in other organisms, other posttranscriptional mechanisms of regulation may be essential for controlling SrbA precursor levels and perhaps the activity of SrbA in A. fumigatus.

It seems evident that many mechanisms of A. fumigatus SREBP regulation remain unknown and may not be fully conserved, compared with the current models of SREBP regulation elegantly described in S. pombe. Thus, future experiments in A. fumigatus will seek to define and better understand the regulation of SrbA and the impact of these mechanisms on fungal virulence and responses to clinically relevant antifungal drugs. For example, elucidating the mechanisms behind the Dsc proteins’ role in SREBP activation in A. fumigatus could perhaps lead to a way to inhibit the SREBP pathway for therapeutic treatment of invasive pulmonary aspergillosis. While SREBPs are conserved in humans, DscD appears to be a fungus-specific protein that, if targeted, could thus potentially avoid the toxicity issues associated with current antifungal drug treatments. Further exploration of the mechanisms of SREBP regulation in pathogenic fungi could thus yield additional fungus-specific drug targets for therapeutic development and new insights into fungal pathogenesis mechanisms.

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