Candida albicans Dap1p Promotes Ergosterol Synthesis via the P450 Protein Erg11p/Cyp51p, Regulating Susceptibility to Azole Antifungal Drugs, Morphogenesis and Damage Resistance

Julia C. Mallory and Rolf J. Craven
Department of Molecular and Biomedical Pharmacology, MS-305 UKMC, Markey Cancer Center, University of Kentucky, Lexington, Kentucky 40536, USA

Abstract: Background: Candida albicans is an opportunistic pathogen in humans and is a significant cause of disease progression and death in immune-compromised patients. One of the most effective drug classes for treating C. albicans infections is the azole drugs which inhibit the P450 protein Erg11p/Cyp51p lanosterol demethylase. Dap1p is part of a widely conserved group of proteins that includes the yeast Dap1p proteins in Saccharomyces cerevisiae and Schizosaccharomyces pombe, the PGRMC1/Hpr6 6/25-Dx proteins in mammals and unnamed homologous in multiple pathogenic fungi. In non-pathogenic yeast and in humans, Dap1p or its homologue activates Erg11p and promotes sterol synthesis. Results: The DAPIp family had not been studied in pathogenic yeast and we studied that deletion of both copies of the DAPIp gene in C. albicans causes a partial arrest in ergosterol synthesis at the step catalyzed by Erg11p. As a result, loss of DAPIp causes elevatedazole drug susceptibility and DAPI is induced by multipleazole drugs. Furthermore, DAPI directs filamentous growth in suspension culture and is required for resistance to the DNA damaging agent Methyl Methane sulfonate (MMS). Conclusions: Present results support a model in which DAPIp activates an essential reaction in pathogenic fungi, suggesting that DAPIp may be a target for antifungal therapeutics to be used in conjunction with existing therapies.

Key words: Candida, DNA damage, sterol, drug resistance, pseudohyphae

INTRODUCTION

Candida infections are a major cause of death in immuno-compromised patients, such as HIV and transplant patients and patients receiving cancer chemotherapy (White et al., 1998). Candida albicans infections are typically treated with azole drugs that inhibit the enzyme Erg11p/Cyp51p lanosterol demethylase (Lopesheva and Waterman, 2007; Waterman and Lopesheva, 2005) or with drugs that the ergosterol biosynthetic pathway. Erg11p is a cytochrome P450 protein (Nebert and Russell, 2002), a class of proteins that was named for its unusual absorption spectrum that arises from bound heme (Werek-Reichhart and Feyereisen, 2000). Erg11p requires a reductase partner and Erg11p activity is elevated by cytochrome b5, Cyp5p (Lamb et al., 1999; Rogers et al., 2004; Schenkman and Janssen, 2003).

Disrupted ergosterol synthesis triggers alterations in the expression patterns of numerous genes (Agarwal et al., 2003; Bannert and Fostel, 2000; Hughes et al., 2000), including 11 ERG (ergosterol synthetic) genes, CYB5/cytochrome b5 (Agarwal et al., 2003), heme synthetic genes and DAPI (damage resistance protein 1). DAPIp is composed largely of a region of limited homology with cytochrome b5 called a heme-1 domain (Hand et al., 2003; Mifsud and Bateman, 2002). Cells deleted for DAPI are defective in ergosterol biosynthesis at the step catalyzed by Erg11p (Hand et al., 2003) and as a result, dapiΔ cells are sensitive to azole antifungal drugs (Hand et al., 2003; Mallory et al., 2005a), which inhibit Erg11p. While DAPIp was originally characterized in S. cerevisiae, DAPIp has a similar role in the phylogenetically distant yeast Schizosaccharomyces pombe (Hughes et al., 2007). DAPIp has been included as part of the global antifungal drug resistance network (Parsons et al., 2004) and likened to a “helping hand” for P450 proteins (Debose-Boyd, 2007).

ERG11 over-expression suppresses itraconazole sensitivity in dapiΔ cells and restores normal ergosterol synthesis to dapiΔ cells (Mallory et al., 2005a) and in some strain backgrounds, DAPIp regulates the stability of

Corresponding Author: Rolf J. Craven, Department of Molecular and Biomedical Pharmacology, MS-305 UKMC, Markey Cancer Center, University of Kentucky, Lexington, Kentucky 40536, USA
Tel: (859) 323-3832 Fax: (859) 257-9608

179
Erg11p (Mallory et al., 2005a). Dap1p binds to heme through a penta-coordinate mechanism that utilizes a carboxy-terminal tyrosine (Ghosh et al., 2005) and heme binding is required for sterol synthesis and for stabilizing Erg11p (Mallory et al., 2005a). In addition to regulating sterol synthesis, Dap1p directs resistance to the methylating agent, methyl Methane sulfonate (Hand et al., 2003). MMS sensitivity is also due to defective Erg11p function (Mallory et al., 2005a), suggesting that sterol synthesis is needed for the repair of chemically induced damage, a conclusion that is supported by genome-wide studies (Bennett et al., 2001). Dap1p is also required for the uptake and storage of iron and like the other Dap1p phenotypes, Dap1p-mediated iron metabolism is mediated by Erg11p (Craven et al., 2007).

In *S. cerevisiae*, Dap1p lacks a putative membrane-spanning sequence and localizes to punctuate cytoplasmic sites that co-fractionate with endosomes (Craven et al., 2007). The localization of *S. cerevisiae* Dap1p overlaps partially with that of Erg11p (Craven et al., 2007) but no direct interaction for the two proteins has been reported. In contrast, *S. pombe* Dap1p has a putative amino-terminal membrane spanning sequence and co-precipitates with Erg11p (Hughes et al., 2007), as does the mammalian Dap1p homologue, PGRMC1/Hpr6 (Hughes et al., 2007). PGRMC1 and the related rodent homologous localize to the endoplasmic reticulum (Crudden et al., 2005; Nolte et al., 2000), with a smaller fractionlocalizing to the cell membrane (Krebs et al., 2000).

Because *S. cerevisiae* and *S. pombe* Dap1p regulate azole drug resistance and iron metabolism, pathways that are important for the biology of pathogenic yeast, we have tested the extent to which *C. albicans* Dap1p performs analogous functions. We have found that, like its relatives in non-pathogenic yeast, *C. albicans* Dap1p regulates ergosterol synthesis at the step catalyzed by Erg11p and promotesazole drug resistance. Furthermore, *C. albicans* Dap1p is required for filamentous growth and for resistance to the DNA damaging agent, methyl methane sulfonate (MMS). Thus, *C. albicans* Dap1p activates an essential pathway in pathogenic fungi that regulates drug susceptibility and damage resistance.

**MATERIALS AND METHODS**

**Culture conditions and chemicals:** Unless stated otherwise, cells were maintained by culturing in Yeast Peptone-Dextrose (YPD) medium at 30°C. Fluconazole (LKT laboratories), ketoconazole (Sigma), itraconazole (Sigma), PD98059 (Calbiochem), methyl, methane sulfonate (Sigma) and ferrozine (Acros) were added at the concentrations indicated in the text.

**Strain construction:** All strain manipulations were performed in the wild-type strain BWP17 (Wilson et al., 1999). The first copy of DAP1 was deleted by targeted integration of the plasmid pJM72 digested with Hind III and Apa I. In all cases, cells were transformed using the standard lithium acetate transformation procedure, except that cells were incubated for 1 h at 30°C in YPD media before plating. We used this approach because we identified unusual recombination events at the 3' end of the DAPI gene when smaller regions of homology were used. We did not detect these events during the deletion of the second copy of DAPI with the HIS3 gene. The disruption of the DAPI gene by URA3 was verified initially by PCR using the primers CaDAP1-1081F and CaURA3-154R for the 5' end and CaURA3+750F and CaDAP1+1600R for the 3' end. Oligonucleotide sequences are shown in (Fig. 1). The DAPI/dap1Δ::URA3 strain was named JMCa3.

The second copy of DAPI was deleted by transformation of JMCa3 with a PCR product generated by amplification of the HIS1 gene from the plasmid pFA-HIS1 (Gola et al., 2003) with the primers CaDAP1-54-KOF and CaDAP1+573-KOR. The deletion of DAPI was verified initially by PCR using the primers CaDAP1-1081F, CaHIS1-280R, CaHIS1+983F and CaDAP1+1600R. The dap1Δ::URA3 dap1Δ::HIS1 strain was named JMCa5. All of the primers are outside the region of homology targeted by the integration. To re-introduce the DAPI gene to dap1Δ/dap1Δ cells, the plasmid pRC65 was digested with BsaI and used to transform JMCa5 cells. Arg' colonies were tested for re-integration using the primers CaDAP1-1081F and CaDAP1+488R-Xho.

**Plasmids:** The CaDAP1 deletion plasmid pJM72 was prepared as follows. (1) The URA3 gene was amplified from pFA-URA3 (Gola et al., 2003) with the primers CaURA3-220F and CaURA3+940R and sub-cloned into the plasmid pCR2.1 (InVitrogen), forming the plasmid pJM65. (2) The 1 kb of genomic DNA flanking the 5' end of DAPI was amplified from BWP17 genomic DNA using the primers CaDAP1-1000F and CaDAP1-1R and sub-cloned into the Hind III and Sac I sites of pJM65, forming the plasmid pJM70. (3) The 1 kb of genomic DNA flanking the 3' end of the DAPI gene was sub-cloned into pCR2.1 using the primers CaDAP1+469F and CaDAP1+1468R, forming the plasmid pJM67. (4) The Not I-Apa I fragment containing the DAPI flanking sequence from pJM67 was sub-cloned into the same sites of plasmid pJM70, forming the plasmid pJM72.

The CaDAP1 knock-in plasmid pRC65 was constructed as follows. The CaDAP1 open reading frame, along with 1000 bp of upstream sequence, was PCR amplified using the primers CaDAP1-1000F-Hind and
CaDAPI+488R-Xho cloned into plasmid pCR2.1 (Invitrogen), resulting in the plasmid pRC63. A NotI fragment containing the ARG4 gene from the plasmid pFA-ARG4 was then cloned into the NotI site of pRC63, forming the plasmid pRC65.

**Spotting assays and plate preparation:** For all spotting assays, cells were serially diluted 1:10 in water and spotted on plates containing 10 μM fluconazole, 0.02% methyl Methane sulfonate, or 100 μM bathophenanthroline. Colonies were photographed after 48 h incubation at 30°C. The growth of the dap1Δ/dap1Δ strain JMCl5 was compared with that of CNC44, an Arg-Ura His` SC5314 derivative (Negredo et al., 1997). For halo formation assays, cells were grown to log phase (approximately A600 = 1), whereupon the A600 was measured. Cells were diluted to 0.3 A600 units in 3 mL of water containing 0.7% of melted Bacto-agar that was maintained at 48°C. The cell suspension was immediately spread on YPD plates and allowed to harden. Paper disks were placed on the agar and 10 μL of various drugs were spotted on the disks. The plates were then incubated for 24-48 h at 30°C and photographed.

**Sterol analysis:** Sterol profiles were analyzed by the KOH/n-heptane extraction procedure of Molzahn and Woods (Molzahn and Woods, 1972) as previously described (Hand et al., 2003). Cells were grown in liquid medium and harvested for sterol analysis at an A600 of 0.5-1. Cells were centrifuged, washed once with H2O and resuspended in 4.5 M KOH/60% ethanol. Cells were then heated at 88-90°C for 1 h in a round-bottom flask. Ethanol (95%) was then added and the cells were heated for an additional hour and cooled to room temperature. The mixture was then extracted with n-heptane and H2O and the n-heptane layer was analyzed by gas chromatography at the University of Kentucky GC-MS facility.

**Morphological analysis:** Approximately 1000 cells mL⁻¹ were plated on YPD containing 10% fetal bovine serum. The plates were incubated at 37°C for 7 days. For filamentous growth, log phase cells were suspended in molten YPS agar (1% yeast extract, 2% bacto-peptone, 2% sucrose and 2% agar) at a concentration of 100 cells/mL and plated. After 1-3 days at 37°C, the colonies were photographed. In other experiments, cells were suspended in spider medium [1% nutrient broth, 0.2% K2HPO4, 1.35% agar and 1% mannitol (Lee et al., 1975)].

**Expression analysis:** RNA was isolated from log phase yeast cells using the RNAasy kit from Qiagen using the manufacturer’s instructions, except that cells were...
spheroplasted in 1 M sorbitol, 100 mM Tris, pH 7.8 and 100 mM EDTA containing 150 µg zymolase. Three micrograms of RNA was reverse transcribed as described previously (Mallory et al., 2005a). The ratios of DAP1:TUB1 (an internal control for cDNA loading) were determined using the primers CaDAP1+2F, CaDAP1+250R, CaTUB1+421F and CaTUB1+600R in the same reaction. PCR reactions were separated on a 1.5% agarose 1000 gel (InVitrogen).

RESULTS

The DAP1 gene conserved among fungi: Dap1p has homologous in virtually all fungi, including pathogenic fungi such as the Candida species, Aspergillus and Cryptococcus (Fig. 1). All of the key sequences in ScDap1p are conserved in this protein family, including the strictly conserved FYGPlxGPyxNFAryxDARSGLA motif at the heart of the heme-binding domain (Fig. 1, center). The S. cerevisiae Dap1p Asp91 and Tyr 138 are required for heme binding and both residues are conserved among all of the fungal Dap1p homologous (Ghosh et al., 2005; Mallory et al., 2005a). However, some Dap1p homologous lack a hydrophobic membrane-spanning sequence at their amino-termini, while others, including CaDap1p, contain this sequence. In this way, CaDap1p resembles its mammalian homologous (Falkenstein et al., 1996; Gerdes et al., 1998; Krebs et al., 2000; Meyer et al., 1998; Noote et al., 2000; Selmin et al., 1996).

The entire open reading frames of each copy of DAP1 were replaced with the URA3 and HIS1 genes, resulting in the dap1Δ::URA3/ dap1Δ::HIS1 strain JMCa5

**Group A-TM domain sub-family**

C. alb.: MTT LELLIYARNLY NPYINPFPSSFLDVSTKSTI PSQGTM TCMKTDK
A. nid.: MS14 TPENLPPLPAFLV YQPMPKPPYPVAPI VPKTOKLPLPPENPYPV
A. fum.: MSIA TPENLPLPAFLV YNPQKAPAVLEAPPVMPFVPFQK
C. neo.: MSLNFP INLLFLPLFLAVYR ILVPPNHPFP (19) EQILAGYDTNQDFPLPIMRVAPPGKI

**Group B-non-TM sub-family**

S. cere.: MTE LIPEPCPVNTSSTESLG-NNASLTNDENGSSPSVAGCJGHLEAKLGLEK
C. glab.: MTEG LIPEPCPVNTSSTESLG-NNASLTNDENGSSPSVAGCJGHLEAKLGLEK

**Group A**

C. alb.: FIVQCKVTGVIYEGVAPPQGTVPQKMKTVNLSTSRMVEEDLDQGKGEK
A. nid.: YIVQCKVTGVIYEGVAPPQGTVPQKMKTVNLSTSRMVEEDLDQGKGEK
A. fum.: YIVQCKVTGVIYEGVAPPQGTVPQKMKTVNLSTSRMVEEDLDQGKGEK
C. neo.: FIVQCKVTGVIYEGVAPPQGTVPQKMKTVNLSTSRMVEEDLDQGKGEK

**Group B**

S. cere.: NTEQKMHCGSKC TPQKMKTVNLSTSRMVEEDLDQGKGEK
C. glab.: NTEQKMHCGSKC TPQKMKTVNLSTSRMVEEDLDQGKGEK

**Group A**

C. alb.: AVEKK GEGCVPK
A. nid.: AUGYK GEGCVPK
A. fum.: EFGYK GEGCVPK
C. neo.: PSETK GEGCVPK

**Group B**

S. cere.: KTEQKMHCGSKC TPQKMKTVNLSTSRMVEEDLDQGKGEK
C. glab.: KTEQKMHCGSKC TPQKMKTVNLSTSRMVEEDLDQGKGEK

Fig. 1: Dap1p is part of a highly conserved family of fungal proteins. Proteins are aligned in two groups based on the presence of an amino-terminal putative trans-membrane sequence. Candida albicans Dap1p is aligned with homologous from Aspergillus nidulans, Aspergillus fumigatus and Cryptococcus neoformans, while the Saccharomyces cerevisiae homologue is aligned with Candida glabrata Dap1p. Residues that are shared by both groups are shown in dark gray, while residues shared within either group are highlighted in light gray. The amino-terminal putative trans-membrane sequences and the central heme-1 domain homologies are boxed and asterisks mark residues that are required for heme binding.
Fig. s2: Deletion of both copies of the *C. alicans* DAP1 gene. One copy of DAP1 (a) was initially deleted by integrating the plasmid pJM72, containing 1 kb of DNA flanking DAP1 open reading frame adjacent to URA3 (b) resulting in strain JMCa3 (c) the second copy of DAP1 was then deleted using a PCR product consisting of the HIS1 gene adjacent to 100 bp of DNA adjacent to the 5' and 3' ends of the DAP1 open reading frame. The *dap1Δ/dap1Δ* strain is called JMCa5 (d) the deletion of DAP1 was tested by PCR using primers within the inserted auxotrophic marker (H-F and H-R for HIS1 and U-F and U-R for URA3) and outside the region of homology (grey stippled line) used to target the integration event (primers D-F and D-R). The panels in (f) show PCR products using the primers shown in part (e) with wild-type (wt) DNA from the strain BWP17 and *dap1Δ/dap1Δ* DNA from the strain JMCa5 as template. The JMCa5 strain was subsequently used for analyzing azole drug sensitivity, ergosterol synthesis and invasive phenotypes.

Fig. 2 (a-c): Mutants lacking Dap1p have altered levels of sterol metabolites. (a) Depiction of the sterol biosynthetic pathway showing the relevant intermediates. (b) The sterol profiles of wild-type CNC44 or *dap1Δ/dap1Δ* JMCa5 and (c) cells were analyzed by gas chromatography, showing increased peaks for lanosterol, 24-methylene lanosterol, ergosta-5,7-dienol and episterol in the *dap1Δ/dap1Δ* strain. For b and c, the X axis represents retention time and the Y-axis represents relative abundance.
The \textit{C. albicans} dap1Δ/dap1Δ mutant has increased azole susceptibility: The dap1Δ/dap1Δ strain JMCa5 were measured using gas chromatography. Ergosterol was synthesized via a multi-step pathway that includes the first sterol intermediate, lanosterol (Fig. 2a). Compared to the wild-type strain (Fig. 2b), JMCa5 exhibited a marked increase in lanosterol and a smaller increase in 24-methylene lanosterol (Fig. 2c), suggesting a partial arrest at the step of ergosterol synthesis catalyzed by Erg11p. In addition, dap1Δ/dap1Δ cells accumulated the Erg5p and Erg3p substrates ergosta-5,7-dienol and episterol, respectively (Fig. 2c).

The \textit{C. albicans} dap1Δ/dap1Δ strain JMCa5 grew poorly on plates containing 2-20 μM fluconazole compared to the strain CEN44 (Fig. 3a) which was used because its auxotrophy is identical to JMCa5 (Negredo et al., 1997). In addition, dap1Δ/dap1Δ cells were hyper-sensitive to the Erg11p inhibitors itraconazole and ketoconazole in spotting assays (Fig. 3a, respectively) and in halo-forming assays for fluconazole (Fig. 3b). In a liquid growth assay, the dap1Δ/dap1Δ strain had a 9-fold lower MICₐₚ for fluconazole than the wild-type strain (2.0 versus 18.2 μM), which was highly significant (P = 6×10⁻⁵, two-tailed t-test). Microscopic analysis revealed enlarged, elongated buds after fluconazole treatment in the dap1Δ/dap1Δ strain that were not detected in wild-type cells (Fig. 3c). The fluconazole sensitivity of the dap1Δ/dap1Δ strain was complemented by the insertion of the wild-type DAPI1 gene (Fig. 3b). Thus, like its \textit{S. cerevisiae} and \textit{S. pombe} analogues, \textit{C. albicans} strains lacking DAPI are sensitive to azole drugs due to aberrant ergosterol synthesis (Hand et al., 2003; Hughes et al., 2007).

We identified the role for \textit{S. cerevisiae} DAPI in ergosterol synthesis, in part, through its transcriptional induction byazole antifungal drugs. We used PCR to test the expression of \textit{C. albicans} DAPI after treatment with variousazole drugs for 3 h and detected a 1.7-2.0-fold induction in the DAPI transcript after treatment with fluconazole, itraconazole and ketoconazole (Fig. 4a). No DAPI band was detectable in cDNA from the dap1Δ/dap1Δ strain JMCa5 (Fig. 4a) and primers for the \textit{C. albicans} tubulin gene TUB1 were included in each reaction to control for sample loading (Fig. 4a). DAPI regulation has been reported to be under the control of the MAP kinase Hog1p and the addition of a MAP kinase inhibitor, PD98059, inhibited the induction of DAPI1 from 1.9-fold with fluconazole to baseline levels (Fig. 4b). These results suggest that MAP kinases may contribute to the regulation of DAPI by azole drugs.

Dap1p regulates filamentous growth: Because Dap1p regulates sterol synthesis in \textit{C. albicans}, we tested the ability of dap1Δ/dap1Δ mutants to undergo filamentous growth. The dap1Δ/dap1Δ strain grew at a wild-type rate under normal growth conditions and appeared morphologically normal microscopically, similar to analogous strains in other yeast species. JMCa5 did not exhibit temperature sensitivity or defective growth on synthetic media.
Fig. 3 (a-f): Dap1p-associated azole susceptibility phenotypes. Wild-type (a) and dap1A (b) were tested for azole susceptibility by halo formation assay in which paper disks were saturated with 5 l of 10 M fluconazole. The dap111 strain had decreased residual growth after treatment. The morphology of the wild-type CNC44 strain changed little after 3 h of fluconazole treatment (c and d), while the dap1A strain JMCa5 developed an increased number of elongated cells (e and f).

Fig. 4 (a-b): DAPI transcription is induced by azole drugs. (a) DAPI expression in the wild-type SC5314 strain after 3 h of 10 μM fluconazole (lane 2), 10 μM itraconazole (lane 3) or 10 μM ketoconazole (lane 4) treatment. Expression was measured by reverse-transcriptase PCR using primers to the TUB1 gene in the same PCR reaction as controls for cDNA loading (lower bands). For lanes 1-4, the DAPI:TUB1 ratios were 0.21, 0.36, 0.37 and 0.41, respectively. The dap1Δ/dap1Δ strain JMCa5 was used to test the identity of the DAPI band (lanes 5 and 6), before or after the same treatments as were used in lane 2. (b) DAPI induction by 10 μM fluconazole after 3 h treatment (lane 2) was reversed by the addition of the MAP kinase inhibitor PD98059 at 10 μM (lane 4), while 10 μM PD98059 had no effect by itself (lane 3). For lanes 1-4, the DAPI:TUB1 ratios were 1.11, 2.13, 1.47 and 1.40, respectively. The analysis was performed using the same conditions described in (a).

MMS sensitivity and low iron growth: Dap1p mediates resistance to the DNA damaging agent MMS in S. cerevisiae. The dap1Δ/dap1Δ strain JMCa5 was markedly sensitive to MMS compared to the wild-type CNC44 strain (Fig. 6a). On the iron chelating agent ferrozine, wild-type strains grew normally and retained their white color, while dap1Δ/dap1Δ strains became dark red, although their growth was not affected (Fig. 6b).
**Pharmacologia 3 (7): 179-189, 2012**

Fig. 5 (a-f): *C. albicans* cells lacking DAPI have an altered morphology. The wild-type strain CNC44 (a, c and e) or the dap1Δ/dap1Δ strain JMCa5 (b, d and f) were plated on media containing 10% serum (a and b) or were resuspended in YPS (yeast-peptone-sucrose, c and d) or spider media (e and f).

Fig. 6 (a-b): MMS sensitivity in cells lacking Dap1p. (a) The wild-type CNC44 strain or the dap1Δ/dap1Δ strain JMCa5 were plated on YPD plates without (rows 1-2) or with 0.015% MMS (rows 3-4) and (b) The same strains were plated on YPD without (rows 5-6) or with 800 μM ferrozine (rows 7-8)
DISCUSSION

*C. albicans* Dap1p is the first member of the Daplp/PGRMC1 family to be characterized in pathogenic fungi. *C. albicans* cells lacking Dap1p accumulate lanosterol, suggesting that Dap1p is required for wild-type Erg11p function and are hyper-sensitive to inhibitors of the ergosterol biosynthetic pathway, like comparable strains in *S. cerevisiae* and *S. pombe* (Hand et al., 2003; Hughes et al., 2007; Mallory et al., 2005a). It is possible that azole susceptibility is due, in part, to the elevated levels of lanosterol in dap1Δ strains, rather than decreased ergosterol synthesis. However, the targets of elevated lanosterol in *C. albicans* are largely unknown. In addition to lanosterol, *C. albicans* dap1Δ mutants accumulate ergosta-5, 7-dienol, the Erg5p/sterol C-22 desaturase substrate and episterol, the substrate of Erg3p/sterol C-5 desaturase. This effect was detected in *S. cerevisiae* (Hand et al., 2003; Mallory et al., 2005a), suggesting that this is a conserved function of Dap1p. Both the sterol C-5 desaturases and sterol C-22 desaturases are activated by cytochrome b, with which Dap1p shares homology (Mifraud and Bateman, 2002). Like Erg11p, Erg5p is a P450 protein while Erg3p contains sequences for iron binding and Dap1p has been implicated in the transport or storage of iron (Craven et al., 2007).

In addition to sterol defects and increased azole susceptibility, one of the most pronounced phenotypes associated with Dap1p was diminished filamentous growth. In the early stages of the assay, filamentous growth was notably absent (Fig. 4) and although dap1Δ colonies were ultimately able to form mycelia, the filamentous structures were smaller than the wild-type strain and contained clusters of unbranched cells. Ergosterol synthesis is important in hyphal formation (Martin and Konopka, 2004). The association between Dap1p and invasiveness may be important for mammalian cells, because the Dap1p homologue, PGRMC1, is over-expressed in clinical tumor samples (Crudden et al., 2005; Irby et al., 2005) and invasiveness is critical in cancer formation. Furthermore, PGRMC1 is expressed in neuronal cells following damage (Guennoun et al., 2007; Laboumarda et al., 2003) and may contribute to the migration and morphology of those cells.

The best characterized function of the Daplp proteins is heme binding. The Dap1p/PGRMC1 proteins were originally identified as progesterone binding proteins but progesterone binding was not detected for recombinant forms of these proteins (Min et al., 2005). In contrast, multiple labs have reported heme binding for Dap1p/PGRMC1 and in a particularly elegant series of studies, *S. cerevisiae* Dap1p was shown to bind to heme through a 5-coordinate mechanism that utilizes a conserved carboxy-terminal tyrosine residue (Ghosh et al., 2005). Heme binding has also been reported for the S. pombe (Hughes et al., 2007), rodent (Min et al., 2005) and human (Crudden et al., 2006; Ghosh et al., 2005) homologues. Nonetheless, there is some evidence that this human Dap1p homologue has a role in progesterone signaling, perhaps through a co-precipitating protein (Peluso et al., 2007). We were unable to detect any difference in proliferation or morphology following treatment of wild-type and dap1Δ strains with progesterone.

*S. cerevisiae* and *S. pombe* Dap1p regulate MMS susceptibility and we have shown that *C. albicans* Dap1p shares this activity. In *S. cerevisiae*, MMS resistance can be restored to dap1Δ mutants through over-expression of the heme biosynthetic proteins Hem1p and Hem2p and by adding exogenous heme (Craven et al., 2007; Mallory et al., 2005a), suggesting that MMS may target the heme biosynthetic pathway. Because Erg11p binds heme, one of the ultimate targets of MMS may be Erg11p and we previously showed that Erg11p over-expression suppresses MMS sensitivity in dap1Δ mutants (Mallory et al., 2005a). This activity is likely conserved between yeast and humans, because the human Dap1p homologue, Hpr6/PGRMC1, is induced by DNA damaging agents (Mallory et al., 2005b) and promotes survival from DNA damage (Crudden et al., 2006).

The mechanism through which Dap1p or its homologous activate Erg11p is under investigation. The *S. pombe* and human homologues bind directly to P450 proteins (Hughes et al., 2007), although this has not been demonstrated in other organisms and the human homologue activates P450 activity in an over-expression system (Min et al., 2005). Thompson et al. recently reported that *S. cerevisiae* Dap1p has a reducing activity similar to that of P450 reductases (Thompson et al., 2007). However, Dap1p has a poor affinity for ferrous heme, which is inconsistent with a direct role in redox cycling (Thompson et al., 2007). These results, together with the findings of the current study, suggest that Dap1p represents a novel regulatory mechanism for P450 proteins such as Erg11p. Thus, Dap1p may represent a novel approach to targeting the most tractable pathway in pathogenic fungi.

ACKNOWLEDGMENTS

We thank Dr. Aaron Mitchell, Jesus Pla and Jurgen Wedlund for the kind gift of strains and plasmids for the analysis and Ikhas Ahmed for reading the manuscript. We thank Dr. Jack Goodman for the sterol analysis, Drs.
Pharmacologia 3 (7): 179-189, 2012

Martin Bard and Robert J. Barbug for advice in interpreting the sterol profiles and Dr. Martin Bard for strains and many helpful suggestions for deleting DAPI. This study was supported, in part, by the NIH grants COBRE P20 RR 15592 and BIRCWH K12 DA14040.

REFERENCES


