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Sexual Development in *Cryptococcus neoformans* Requires *CLP1*, a Target of the Homeodomain Transcription Factors Sxi1α and Sxi2**a**▽

Joanne L. Ekena,[1] Brynne C. Stanton,[1] Jessica A. Schiebe-Owens,[2]† and Christina M. Hull[1,2]*

Department of Biomolecular Chemistry[1] and Department of Medical Microbiology & Immunology,[2] University of Wisconsin, School of Medicine and Public Health, Madison, Wisconsin 53706

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Sexual development in the human fungal pathogen *Cryptococcus neoformans* is a multistep process that results in the formation of spores, the likely infectious particles. A critical step in this developmental process is the transition from bud-form growth to filamentous growth. This transition is controlled by the homeodomain transcription factors Sxi1α and Sxi2a, whose targets are largely unknown. Here we describe the discovery of a gene, *CLP1*, that is regulated by Sxi1α and Sxi2a and is essential for sexual development. In vitro binding studies also show that the *CLP1* promoter is bound directly by Sxi1α and Sxi2a. The deletion of *CLP1* leads to a block in sexual development after cell fusion but before filament formation, and cells without *CLP1* are unable to grow vegetatively after cell fusion. Our findings lead to a model in which *CLP1* is a downstream target of the Sxi proteins that functions to promote growth after mating and to establish the filamentous state, a critical step in the production of spores.

Cryptococcus neoformans is an environmental fungus that has the capacity to infect and cause disease in mammalian hosts (6). Immunocompromised individuals, such as people with AIDS, chemotherapy patients, and those undergoing immunosuppressive therapies, are most at risk of developing cryptococcal meningitis. Infection appears to occur through a respiratory route in which *C. neoformans* particles are inhaled and lodge in the lung alveoli. The likely infectious particle in *C. neoformans* infections is the spore. One mechanism by which spores can be produced is sexual development (1, 13). During this process, cells of opposite mating types (**a** and α) sense one another, fuse cytoplasms (but not nuclei), and form filaments. Each filament cell contains two nuclei of opposite mating types that remain distinct until the terminal filament cell forms a specialized structure called a basidium. In the basidium, nuclear fusion and meiosis take place, and their products are repeatedly replicated, packaged, and expressed as buds on the surface of the basidium in four long chains of spores (18). The formation of filaments is a critical step in the spore formation process; dikaryotic filaments are largely aerial, and moving spores off solid substrates is likely critical for dispersal (1). Dikaryons are common among basidiomycete fungi, the phylum to which *C. neoformans* belongs (7). This phylogenetic group includes the corn smut, *Ustilago maydis*, as well as most mushrooms, including the ink cap mushroom, *Coprinopsis cinerea* (5, 17). Fungi in this group have very different life cycles, but those that have been studied in detail all include a phase in which filament cells grow with distinct nuclei prior to developing fruiting bodies and spores. This critical phase allows host cell penetration in the case of *U. maydis* and the ability to forage for nutrients in *C. cinerea* (5, 17). By and large, however, the genes required for this stage of development are not known. What is known is that the dikaryotic transition is controlled by homeodomain transcription factors (15). In *U. maydis*, the proteins bE and bW must interact with one another to initiate dikaryon formation (24). Similar proteins, HD1 and HD2, control the process in *C. cinerea*, and the Sxi1α and Sxi2**a** proteins are required in *C. neoformans* (11, 16).

Efforts to determine potential targets of Sxi1α and Sxi2**a** resulted in the identification of a gene with similarity to the *clampless* gene of *C. cinerea*. With *C. cinerea*, Inada et al. carried out a screen for dikaryons that could not form clamp cells, specialized cells required for proper nuclear migration (14). They identified a mutant in which clamp cells were not formed and named it the *clampless-1* mutant. Cloning and sequencing of the *clampless* gene revealed a predicted protein with no identifiable structural features and a predicted promoter region with putative homeodomain binding sites conforming to the sequence $GATGN_xACA$, suggesting direct regulation by HD1 and HD2. A *CLP1* gene was also identified in *U. maydis* and is required for clamp cell formation as well as sexual development (23).

Based on sequence similarity to the Clp1 proteins from *U. maydis* and *C. cinerea*, we identified a putative Clp1 protein in *C. neoformans* and investigated its potential role in *C. neoformans* sexual development. We found that the *CLP1* homolog in *C. neoformans* is essential for the formation of dikaryotic filaments during sexual development, functions after cells of opposite mating types fuse, and is regulated by Sxi1α and Sxi2**a**. Along with other data presented here, these findings lead to a model of sexual development in which *CLP1* is required for cells to initiate growth after cell fusion.

MATERIALS AND METHODS

Strain manipulations and media. All strains used were of the serotype D background, and their genotypes are given in Table 1. All were handled using

* Corresponding author. Mailing address: University of Wisconsin, Madison, 587 Medical Science Center, 1300 University Avenue, Madison, WI 53706. Phone: (608) 265-5441. Fax: (608) 262-5253. E-mail: cmhull@wisc.edu.

† Present address: Department of Ophthalmology, University of Iowa, Carver College of Medicine, 375 Newton Road, 4111 Medical Education & Research Facility, Iowa City, IA 52242.

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TABLE 1. Strains used in this study

Strain	Genotype	Reference
JEC20	**a**	19
JEC21	α	19
CHY1069	**a** *clp1*Δ::*URA5*	This study
CHY1072	α *clp1*Δ::*URA5*	This study
CHY1090	**a** *ura5*::Neo^r	This study
CHY1093	α *ura5*::Neo^r	This study
CHY1177	α *clp1*Δ::Neo^r *ura5*	This study
CHY1179	**a** *clp1*Δ::Neo^r *ura5*	This study
CHY1421	α *clp1*Δ::Nat^r	This study
CHY1187	α *clp1*Δ::Neo^r *ura5 CLP1-URA5*	This study
CHY610	α *sxi1*αΔ::*URA5*	12
CHY768	**a** *sxi2***a**Δ::*URA5*	11
CHY1345	**a** Nat^r	This study
CHY1348	α Neo^r	This study
CHY1422	**a** *clp1*Δ::Neo^r *ura5 CLP1-URA5*	This study
CHY875	**a**/α *ade2*/+ *ura5*/+ *lys1*/+	This study
CHY1457	**a**/α *clp1*Δ::Neo^r/*clp1*Δ::Nat^r *ura5*/+	This study
CHY1458	**a**/α *clp1*Δ::Neo^r/*clp1*Δ::Nat^r *ura5*/+	This study
CHY1459	**a**/α *clp1*Δ::Neo^r/*clp1*Δ::Nat^r *ura5*/+	This study

standard techniques and media as described previously (2, 25). Sexual development assays were conducted on V8 medium at room temperature in the dark for 2 to 4 days. Sexual development was evaluated by observing the periphery of test spots on V8 medium. The mating tester strains used were JEC20 (**a**) and JEC21 (α) (19). For confrontation assays, strains were streaked on yeast extract-peptone-dextrose (YPD) agar and incubated for 2 days and then streaked near one another on low-nitrogen agar and incubated at room temperature in the dark for 4 days before they were photographed. Fusion assays were carried out by resuspending cells at 1×10^8 cells/ml and mixing equal numbers of mating partners. Ten microliters of each mix was spotted onto a V8 agar plate, and plates were incubated at room temperature in the dark. After 24 h, the cells were scraped off the V8 plates, resuspended in 100 μl water, and spread on minimal medium (SD) containing 200 μg/ml G418. Plates were incubated at 30°C for 5 days, and the resulting colonies were counted.

Constructing *clp1* deletion strains. *clp1*Δ constructs contained either a *URA5*, neomycin resistance (Neo^r), or nourseothricin (Nat^r) cassette flanked by ~1 kb of sequence from upstream and downstream of the *CLP1* open reading frame (ORF). The *CLP1* 5′-flanking region was amplified with primers CHO624 (5′-GACGGTACCGGATCCAATAAGCATGGCAACTGTG-3′) and CHO625 (5′-GACACTAGTGGCGAATGGATGGAAGGATG-3′), and the 3′-flanking region was amplified with CHO626 (5′-GACGCGGCCGCATAGAAAAGTCA TTACTCATC-3′) and CHO627 (5′-GACCTCGAGGGATCCCTCTCCTTGG CGAGGGCAAG-3′). PCR fragments were cloned into pCR2.1, using a TOPO TA cloning kit (Invitrogen). One *CLP1* disruption construct (*clp1*::*URA5*) was made by cloning the 5′ flank and the 3′ flank into a plasmid containing a *URA5* cassette to create pCH317. The second disruption construct (*clp1*::Neo^r) was made by replacing the *URA5* cassette of pCH317 with the Neo^r cassette from pJF1 (pCH361) to make pCH318. A third deletion cassette (*clp1*::Nat^r) was made by replacing the *URA5* cassette of pCH317 with the Nat^r cassette from pCH233 (Nat^r cassette in pCR2.1) to make pCH574. The *clp1*::*URA5* and *clp1*::Neo^r deletion cassettes were transformed into serotype D *ura5* strains JEC34 (**a**) and JEC43 (α) by biolistic transformation, grown on medium containing 1 M sorbitol, and selected on medium either lacking uracil or containing 200 μg/ml G418 (28). The *clp1*::Nat^r cassette was transformed into serotype D strain JEC21 (α) and selected on medium containing 200 μg/ml nourseothricin. Transformants were screened by PCR for the proper integration of the deletion construct, and positive clones were confirmed by Southern blot analysis (4). The resulting independent *clp1*::*URA5* strains were designated CHY1069 (**a**) and CHY1072 (α), the *clp1*::Neo^r *ura5* strains were designated CHY1179 (**a**) and CHY1177 (α), and the *clp1*::Nat^r strain was designated CHY1421 (α).

Construction of *CLP1*-reconstituted strains. A construct for reconstitution of *clp1* deletion strains with *CLP1* was constructed by cloning a fragment containing the entire ORF with ~1-kb flanking sequences into a pCnTEL-URA derivative (pCH390), using BamHI, to create pCH454. The complementation fragment was liberated and transformed into CHY1177 (α *clp1*::Neo^r *ura5*). Transformed cells were selected on SD plates containing 1 M sorbitol and crossed with CHY1069 (**a** *clp1*::*URA5*) to assay for recovery of sexual development. Positive isolates were confirmed via Southern blotting.

Protein expression and purification. A fragment corresponding to the homeodomain of Sxi2**a** cDNA was cloned into the BamHI site of the pRSET-A expression vector (with a six-histidine tag) to create pCH292. Protein expression was carried out in *Escherichia coli* BL21(DE3)/pLysS cells, which were induced in Zymo Research EB/OB medium according to the manufacturer's instructions. Proteins were purified by resuspending induced cells in histidine binding buffer (500 mM NaCl, 20 mM Tris, pH 8.0, 5 mM imidazole, 50 mM phenylalanine, 50 mM isoleucine) containing a protease inhibitor cocktail and Igepal detergent (0.01%), sonicating them, and centrifuging them at 4°C for 30 min at 14,000 rpm. The supernatant was equilibrated with Qiagen nickel resin with gentle agitation and applied to a gravity-flow column at room temperature. The resin was washed with histidine binding buffer, and proteins were eluted with histidine elution buffer (500 mM NaCl, 20 mM Tris, pH 8.0, 0.5 M imidazole, 50 mM phenylalanine, 50 mM isoleucine). The eluate was concentrated using a Microcon microconcentrator according to the manufacturer's instructions.

EMSA. To generate radiolabeled probes for electrophoretic mobility shift assays (EMSAs), PCRs with mixtures containing [α-^{32}P]dCTP were performed. To generate probes, a plasmid containing 1 kb of genomic DNA upstream of the *CLP1* ORF (pCH320) was used as the template in combination with the following primers: for probe A, CHO628 (5′-CGGAATTATGCATTCCTGCTTGCC CGACGGC-3′) and CHO629 (5′-CGGAATTGCTGGAGTTGACGAGCAAA TGAAG-3′); for probe B, CHO572 (5′-CGGAATTCGCATTGACGGATGAG TGG-3′) and CHO605 (5′-AATAAAAGTGTCTGTAAGTGAAGGC-3′); and for probe C, CHO573 (5′-CGGAATTCGGCGAATGGATGGAAGGATG-3′) and CHO604 (5′-CACGACGCAAGGACCTTGCACGCCGCCT-3′). The PCR-generated probes were purified in a nondenaturing polyacrylamide gel. The resulting probes were used in binding reactions in a buffer containing 100 mM NaCl, 0.5 mM EDTA, 60 mM Tris-Cl, pH 7.9, 5 mM $MgCl_2$, 5 mg/ml bovine serum albumin, 50 μg/ml poly(dI-dC), and 10% glycerol. Probes and the purified Sxi2**a** homeodomain were incubated at 4°C for 30 min. Reaction mixtures were electrophoresed in a 5% nondenaturing polyacrylamide gel (200 V for 3.5 hours) at 4°C. Gels were dried and exposed to Kodak film to visualize shifts.

Northern blot analysis. RNAs were prepared from *C. neoformans* cells by using a hot phenol method (4). Strains were grown on solid V8 medium for 24 h at room temperature before they were harvested by scraping off the agar surface. Northern blots were carried out according to standard protocols, with 10 μg of total RNA used for each sample (4). The glycerol-3-phosphate dehydrogenase gene (*GPD1*) probe was generated by PCR, using CHO651 (5′-CGTCGTTGA ATCTACCGGTG-3′) and CHO652 (5′-CACCAGCAATGTAAGAGATG-3′), and radiolabeled probes (Decaprime II kit; Ambion) were used in hybridization reactions at 65°C as described previously (9). A *CLP1* riboprobe template was made by amplifying a 450-bp fragment from genomic DNA, using CHO834 (5′-AGTCATCTAGAATGGCTACTTACCTTGCTCCTCCGGTCTC-3′) and CHO835 (5′-TGACAGGCATGCTTCAATTGAAGGGAACCTAGGATAAG GC-3′); digesting the resulting product; and cloning it into pDP18 (Ambion). Radiolabeled riboprobe was generated using α-^{32}P-labeled UTP and T7 polymerase, using a Maxiscript SP6/T7 kit (Ambion) according to the manufacturer's protocol. Hybridizations and washes were carried out according to the manufacturer's instructions.

Sequence manipulations. Sequence comparisons were conducted against the *C. neoformans* genome sequence (*C. neoformans* Genome Project; Stanford Genome Technology Center and The Institute for Genomic Research) by using the BLAST algorithm (3). The *C. neoformans CLP1* sequence can be accessed in GenBank under accession number BK006302.

Microscopy and staining. Light microscopy was carried out on a Zeiss Axioplan microscope fitted with a 20× long-working-distance objective. Photographs were taken with a Nikon Coolpix 5400 camera mounted on the microscope. Fluorescence microscopy was carried out with a Zeiss Axioskop 2 fluorescence microscope fitted with an Axiocam MRM REV3 digital camera and corresponding AV4 software. For staining of fusion events and filaments, cells from crosses were plated on a thin layer of V8 medium on a microscope slide and incubated at room temperature for 24 h. Cells were fixed in 10% formaldehyde–1× phosphate-buffered saline (PBS) for 20 min, washed three times with PBS, permeabilized with 1% Triton X-100–1× PBS for 10 min, and washed three times with PBS. To visualize septa and nuclei, cells were incubated in 1× PBS containing 0.4 μg/ml calcofluor white and 1 nM Sytox green for 20 min, washed three times with PBS, and mounted on slides in 50% glycerol.

5′ and 3′ RACE. Rapid amplification of cDNA ends (RACE) was performed on RNAs isolated from crosses between wild-type strains JEC20 and JEC21 and *sxi*Δ deletion strains CHY610 (*sxi1*αΔ) and CHY768 (*sxi2***a**Δ). Both 5′ and 3′ RACE were performed using a First Choice RLM RACE kit from Ambion

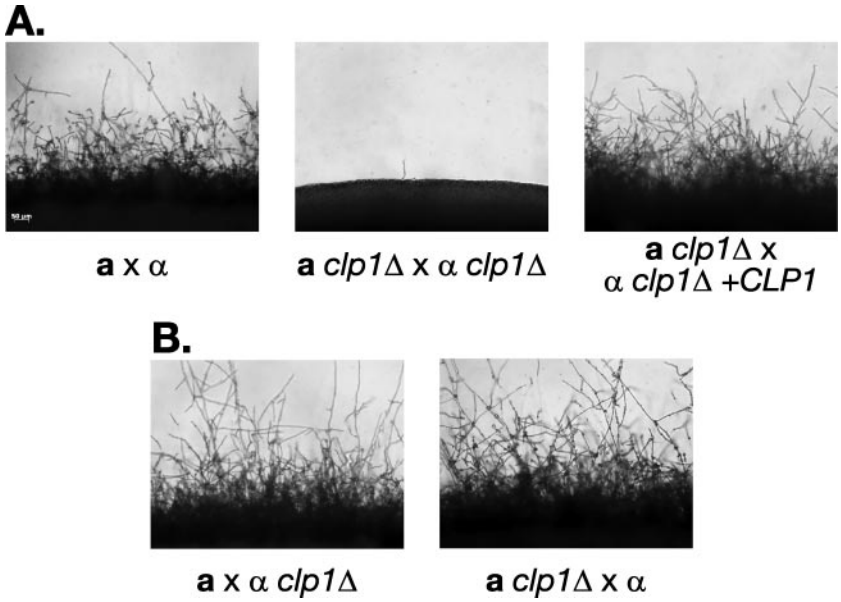

FIG. 1. *CLP1* is required for sexual development. (A) *clp1* deletion and reconstitution phenotypes. (Left) Sexual development of a cross between wild-type **a** and α strains (JEC20 × JEC21). (Middle) Cross between two representative **a** *clp1*Δ and α *clp1*Δ strains (CHY1069 × CHY1072). (Right) Cross between an **a** *clp1*Δ strain and a representative α *clp1*Δ strain containing an ectopically integrated copy of the *CLP1* gene under the control of its own promoter (CHY1069 × CHY1187). (B) Phenotypes of unilateral *clp1*Δ crosses. (Left) Cross between a wild-type **a** strain and an α *clp1*Δ strain (JEC20 × CHY1072). (Right) Cross between an **a** *clp1*Δ strain and a wild-type α strain (CHY1179 × JEC21). All assays were carried out on V8 juice agar at room temperature in the dark and photographed after 48 h.

according to the manufacturer's instructions. The gene-specific primers for 5′ RACE were CHO922 (5′-ATATCGCTGAGGGCGATGTTAGGT-3′ [outer primer]) and CHO923 (5′-ACTGGATCCTAGAAGAGACCGGAGGAGCAA GGTAA-3′ [inner primer]). Gene-specific primers for 3′ RACE were CHO975 (5′-CAGAGGTCGAGTTCATTGACC-3′ [outer primer]) and CHO976 (5′-CG AGACCTGTCAAGGACGAC-3′ [inner primer]). PCR fragments of the predicted sizes were TOPO cloned into pCR2.1 (Invitrogen) and sequenced. Resulting sequences were compared with the genomic *CLP1* sequence.

Construction of *clp1*/*clp1* diploids. Diploids were created by mixing differentially marked strains, spotting them on V8 medium for 24 h, plating them on selective medium, and incubating them at 37°C for several days. Diploids with both copies of *CLP1* deleted were made by crossing CHY1422 (*clp1*Δ), which contains pCH454 (pCLP1), with CHY1421 (*clp1*Δ). Diploids were selected on YPD containing 100 μg/ml G418 and 100 μg/ml nourseothricin. Colonies that grew were confirmed as diploids by PCR for *SXI1*α, using primers CHO408 (5′-CGAAGGGCAAAGTCGAAAACG-3′) and CHO409 (5′-CCGAAATAAT GGGAACTCC-3′), and for *SXI2***a**, using CHO500 (5′-ATGGGCAGCAACCT TGACATC-3′) and CHO534 (5′-TGTTTTCTTGCCTCGATTCCT-3′). Strains were assayed for loss of the pCLP1 plasmid by PCR for *CLP1*, using primers CHO662 (5′-GCCAAAGCACGAGGACG-3′) and CHO663 (5′-CCAGAGAT GGCATCAAG-3′).

RESULTS

Clp1 is required for sexual development. Based on the sequences of the *Coprinopsis cinerea* and *Ustilago maydis* Clp1 proteins (14, 23), we identified a putative Clp1 homolog in *C. neoformans*. Although the overall sequence similarity is low (~23% over 472 amino acids), there is a carboxy-terminal region of ~60 residues with sequence similarity of ~35%, suggesting a significant relationship between the protein sequences. In an analysis of the *U. maydis* Clp1 protein, Scherer et al. identified the same potential homolog from *C. neoformans* and also designated it Clp1 (23). The Clp1 proteins do not have any identifiable domains or features, and no proteins with similarity to Clp1 can be identified outside the basidiomycete fungi. Similarities to the other basidiomycete proteins prompted us to investigate the role that a Clp1 protein might play in *C. neoformans*.

We began by deleting the predicted ORF for *CLP1* from both mating types (**a** and α), complementing each deletion strain with a wild-type copy of the gene, and assaying all strains for phenotypes under an array of conditions. Multiple, independent *clp1* deletion strains exhibited no discernible phenotypes with respect to haploid growth under any of the conditions tested (data not shown); however, they did have profound defects in sexual development. When strains containing deletions of *CLP1* in both **a** and α cells were crossed with one another under conditions favorable for sexual development (e.g., growth on V8 juice agar), there was a complete absence of proper sexual development (Fig. 1A), that is, the strains failed to form dikaryotic filaments, basidia, or spores. However, in unilateral crosses (wild-type **a** × α *clp1*Δ cells or **a** *clp1*Δ × wild-type α cells), sexual development was indistinguishable from that of the wild type, suggesting that a single copy of *CLP1* is sufficient to drive sexual development (Fig. 1B). We confirmed that deletion of the *CLP1* gene was responsible for the defect in sexual development by transforming the *clp1*Δ strains with a wild-type copy of the *CLP1* gene and carrying out complementation tests. Four independent transformants confirmed to have a single, randomly integrated copy of *CLP1* by Southern blotting (α *clp1*Δ mutant plus *CLP1*) were crossed with *clp1* deletion strains (**a** *clp1*Δ). Full sexual development was completely restored and was indistinguishable from wild-type sexual development in all cases. A representative transformant is shown in (Fig. 1A). The complementing gene restored sexual development to wild-type levels,

TABLE 2. Fusion assay results[a]

Cross	No. of colonies	Strain designations[b]
a × α	239 ± 84	CHY1090 × JEC21 (marked **a**)
	522 ± 102	JEC20 × CHY1093 (marked α)
a *clp1*Δ × α	196 ± 57	CHY1179 × JEC21 (marked **a**)
	438 ± 32	CHY1069 × CHY1093 (marked α)
a × α *clp1*Δ	300 ± 109	CHY1090 × CHY1072 (marked **a**)
	287 ± 39	JEC20 × CHY1177 (marked α)
a *clp1*Δ × α *clp1*Δ	0	CHY1179 × CHY1072 (marked **a**)
	0	CHY1069 × CHY1177 (marked α)
a × **a**	0	JEC20 × CHY1090 (marked **a**)
α × α	0	CHY1093 × JEC21 (marked α)

[a] Differentially marked strains (Ura^+ Neo^s × Ura^- Neo^r) were mixed under sexual development conditions (V8 for 24 h at 25°C) and then plated on minimal medium containing neomycin. The second column shows the numbers of colonies recovered from selective plates after 3 days of growth at 30°C (carried out in triplicate for each strain pair tested).
[b] The Ura^- Neo^r strain is indicated as "marked" in each case.

consistent with a single copy of *CLP1* being sufficient for sexual development.

***clp1*Δ strains are defective in dikaryotic filament formation.** To understand where the defect in sexual development occurred, we carried out assays to evaluate each stage of sexual development up to the point at which a defect was observed (i.e., mate recognition, cell fusion, and dikaryotic filament formation). To evaluate the capacity of the *clp1*Δ strains to detect a mating partner, we carried out confrontation assays in which wild-type and *clp1*Δ strains were placed near each other on solid medium and evaluated for the ability to either swell (**a** cells) or produce germ tubes (α cells) in response to an opposite mating partner. All combinations of wild-type and *clp1*Δ strains were tested against one another, and there were no detectable defects or changes in the ability of the *clp1*Δ strains to respond to or elicit a response from a mating partner (data not shown). This result suggested that *clp1*Δ strains both make and respond to mating pheromone at levels similar to those of wild-type strains. To assess potential defects in the next step of sexual development, we evaluated the capacity of the *clp1*Δ strains to fuse with a mating partner by carrying out fusion assays. Differentially marked wild-type and *clp1*Δ strains (Ura^- Neo^r × Ura^+ Neo^s) were mixed under sexual development conditions (V8) for 24 h and then plated on selective medium (minimal medium containing neomycin). Only strains that fused with a mating partner would have the ability to form colonies under selection. We observed that *clp1*Δ strains did not have the ability to form colonies in fusion assays, suggesting a defect in cell fusion (Table 2). However, this result was difficult to reconcile with the fact that unilateral crosses (wild type × *clp1*Δ mutant) were indistinguishable from wild-type crosses, suggesting that *CLP1* functions after cell fusion. Upon careful microscopic observation, we found that **a** *clp1*Δ × α *clp1*Δ crosses had a phenotype different from that of strains that simply cannot fuse with one another. As shown in Fig. 2A, wild-type **a** × wild-type α and **a** *clp1*Δ × α *clp1*Δ crosses resulted in elongated, dumbbell-shaped cells that contained two nuclei at early time points. These apparent fusion products have been observed by others and appear to be the result of fusion between cells of opposite mating types (20). These binucleate cells did not form in control crosses between cells of the same mating type, either wild type or *clp1*Δ mutant (data not shown). At later time points, the difference between wild-type and *clp1*Δ strains became apparent. After 17 h under sexual development conditions, wild-type cells fused, and the fusion products had begun to form the next developmental stage, dikaryotic filaments. As shown in Fig. 2B, wild-type cells formed long filaments with two nuclei and characteristic clamp cells. These filaments continued to grow and complete sexual development. In contrast, the *clp1*Δ strains elongated somewhat but did not form dikaryotic filaments. The fusion products arrested as elongated dumbbells and did not continue to grow or divide, suggesting that cell fusion can occur but that outgrowth cannot.

Because *clp1* mutants appeared to be able to fuse but not to undergo sexual development, we tested whether they could form diploids. Diploid strains of *C. neoformans* var. *neoformans* can be outgrown in the laboratory by crossing marked strains and select-

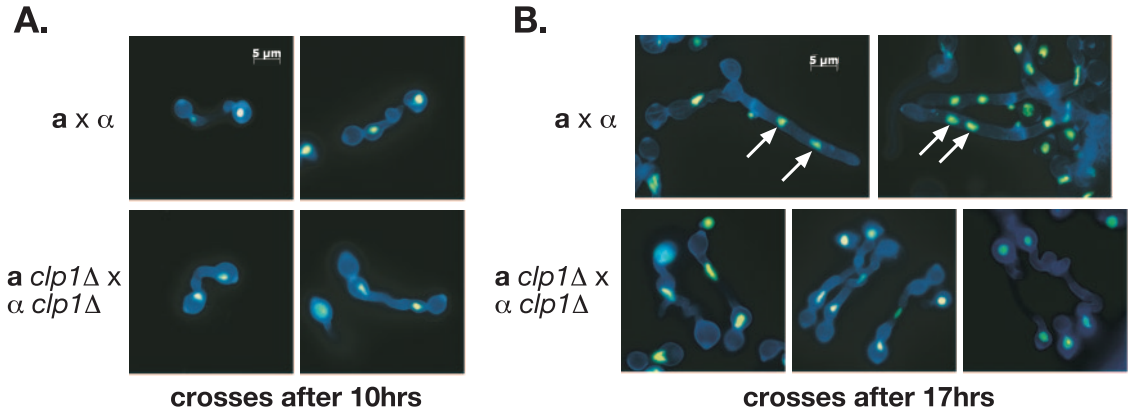

FIG. 2. *CLP1* is required for growth after cell fusion. (A) Wild-type and *clp1*Δ strains show similar phenotypes early in sexual development. All panels show representative fields of fusion products from crosses after 10 h on V8 juice medium. The top two panels show wild-type **a** × α strains; the bottom two panels show **a** *clp1*Δ × α *clp1*Δ strains. (B) *clp1*Δ strains do not progress after cell fusion. All panels show representative fields of cells from crosses after 17 h on V8 juice medium. The top two panels show wild-type **a** × α strains forming dikaryotic filaments; white arrows indicate dikaryotic nuclei in filament cells. The bottom three panels show **a** *clp1*Δ × α *clp1*Δ strains as stalled fusion products; there are no dikaryons visible. The cells in all panels were stained with calcofluor white to visualize the cell walls and Sytox green to visualize the nuclei.

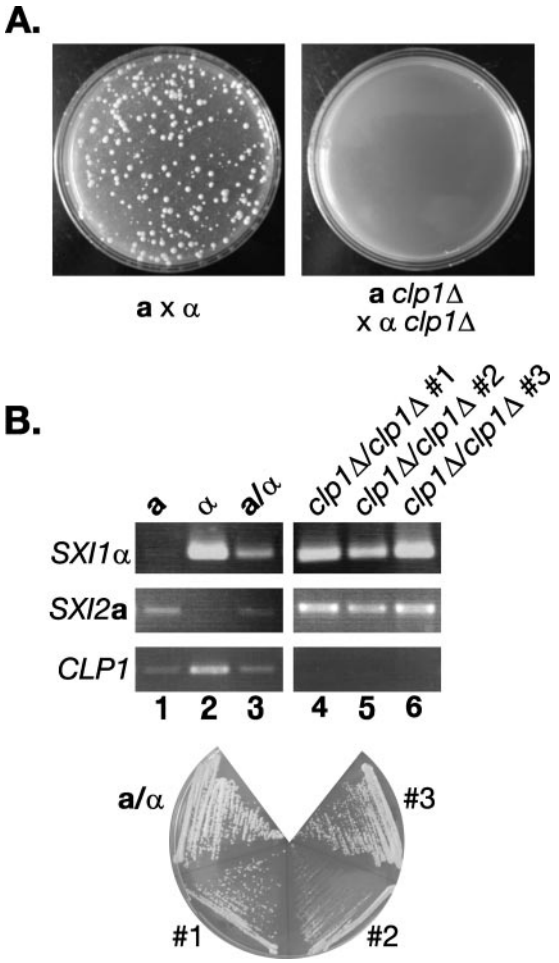

FIG. 3. Diploid formation requires *CLP1*. (A) *CLP1* is required for diploid formation. Crosses between wild-type **a** and wild-type α strains (CHY1345 × CHY1348) and **a** *clp1*Δ and α *clp1*Δ strains (CHY1422 × CHY1421) were carried out on V8 agar for 24 h at room temperature. Resulting mixes were then transferred to selective plates and grown at 37°C to induce diploid formation. Panels show petri plates of diploids formed in each cross. (B) *clp1*Δ/*clp1*Δ diploids grow like wild-type diploids. PCR to assess genotypes was carried out with primers for *SXI1*α, *SXI2***a**, and *CLP1* on genomic DNAs from the indicated strains. Products were visualized in an agarose gel. Lane 1, wild-type **a** haploid (JEC20); lane 2, wild-type α haploid (JEC21); lane 3, wild-type **a**/α diploid (CHY875); lanes 4 to 6, *clp1*Δ/*clp1*Δ diploid strains 1 to 3 (CHY1457 to CHY1459). The petri plate shows growth of wild-type and *clp1*Δ/*clp1*Δ diploids on rich medium for 3 days at 37°C.

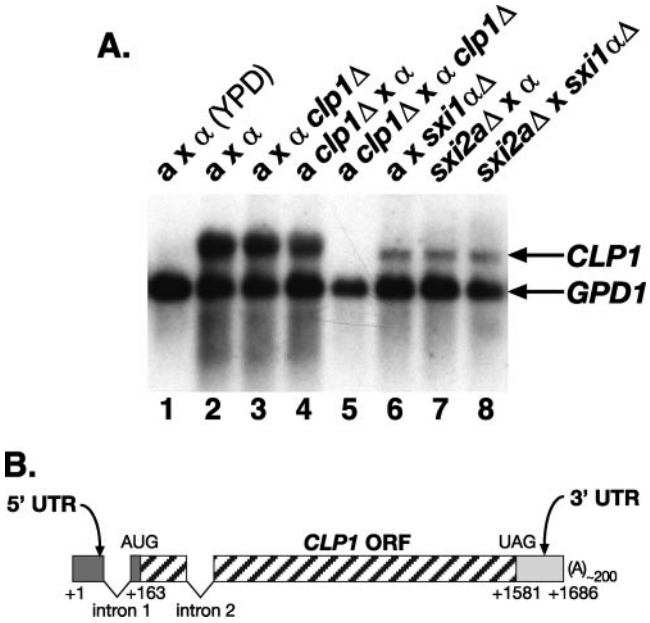

FIG. 4. *CLP1* is regulated by *SXI1*α and *SXI2***a**. (A) The *CLP1* transcript was evaluated by Northern analysis under a variety of growth conditions. RNAs were isolated from the following crosses: lane 1, **a** × α (JEC20 × JEC21) under high-nutrient conditions (YPD); lane 2, **a** × α (JEC20 × JEC21) under sexual development conditions (V8); lane 3, **a** × α *clp1*Δ (JEC20 × CHY1072); lane 4, **a** *clp1*Δ × α (CHY1069 × JEC21); lane 5, **a** *clp1*Δ × α *clp1*Δ (CHY1069 × CHY1072); lane 6, **a** × *sxi1*αΔ (JEC20 × CHY768); lane 7, *sxi2***a**Δ × α (CHY610 × JEC21); and lane 8, *sxi1*αΔ × *sxi2***a**Δ (CHY610 × CHY768). All of the RNAs in lanes 2 through 8 came from crosses carried out on V8 juice medium. Genotypes and conditions are indicated over the lanes. The top arrow indicates the *CLP1* transcript. The bottom arrow indicates the *GPD1* transcript used as a loading and hybridization control. (B) Architecture of the *CLP1* mRNA transcript. Hatched lines indicate the *CLP1* ORF. Shaded regions indicate the 5′ and 3′ UTRs. The total transcript size is 1,686 bases. Two introns are spliced out of the transcript, at positions 141 and 479. Intron 1 is 63 bases in length, and intron 2 is 51 bases in length. Intron 1 resides in the 5′ UTR. The *CLP1* transcript is polyadenylated with a poly(A) tail of ~200 bases.

ing fusion products at 37°C (13). These mononucleate, budding yeast cells can be maintained at 37°C and induced to undergo sexual development at 25°C (26). To test for diploid formation, wild-type and *clp1*Δ strains with different selectable markers were crossed for 24 h on V8 medium and grown under selection at 37°C. Colonies were recovered efficiently from crosses between wild-type strains, but no colonies were formed from crosses of *clp1*Δ strains (Fig. 3A). This inability to form diploids suggests that the *clp1*Δ phenotype is not due to a defect in the ability to form filaments but rather represents an inability to grow after cell fusion. To test this hypothesis, we carried out a selection for diploids with *CLP1* carried on a plasmid in one of the strains. In this cross, diploids were recovered, but only when the strains contained *CLP1* (data not shown). Strains harboring plasmids without *CLP1* were unable to form diploids. These *clp1*Δ/*clp1*Δ(pCLP1) diploids were outgrown to induce plasmid loss, and their genotypes were confirmed by PCR (Fig. 3B). The resulting *clp1*Δ/*clp1*Δ diploids grew like wild-type diploids on YPD, indicating that once the diploids had formed, *CLP1* was not required for diploid growth (Fig. 3B).

***CLP1* transcription is upregulated during sexual development and is controlled by Sxi1α and Sxi2a.** To determine the expression profile of *CLP1*, transcript levels were evaluated under several different conditions. RNAs were isolated from wild-type and *clp1*Δ haploid **a** and α cells as well as from crosses under both high-nutrient (YPD) and sexual development-inducing (V8) conditions. Transcript levels were evaluated by Northern blotting using a probe specific to *CLP1*. The *CLP1* transcript was not detectable in haploid cells under any conditions tested, including sexual development conditions (data not shown). As shown in Fig. 4A, it was also undetectable in crosses under high-nutrient conditions (lane 1). However, under sexual development conditions in crosses between wild-type strains, the *CLP1* transcript was readily detectable, indicating that *CLP1* is induced during sexual

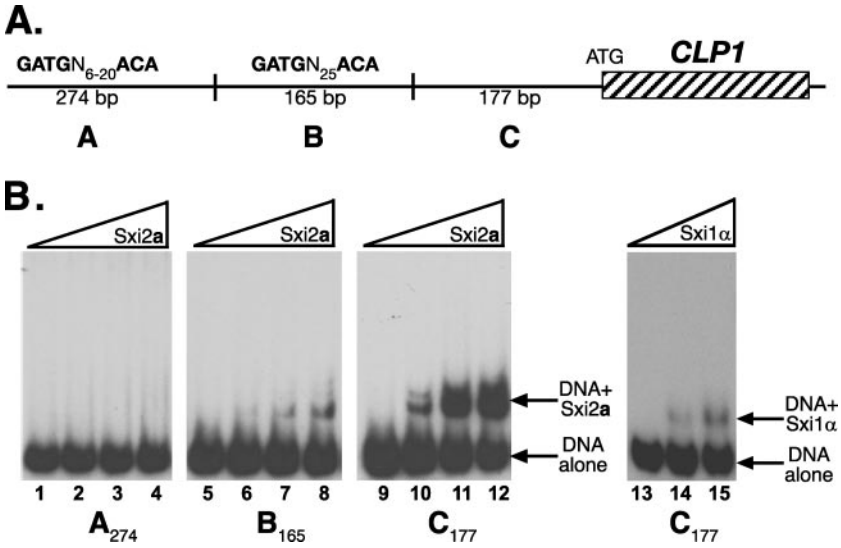

FIG. 5. Sxi1α and Sxi2**a** bind directly to the *CLP1* promoter in vitro. (A) The *CLP1* promoter contains predicted homeodomain binding sites. The hatched box represents the *CLP1* gene in the genome. The black line represents the DNA region upstream of the predicted start codon of the gene. This region is divided into three regions, designated A, B, and C, whose lengths are indicated. The predicted homeodomain binding sites reside in regions A and B. (B) Sxi1α and Sxi2**a** bind fragment C. Fragments of Sxi2**a** and Sxi1α proteins purified from *E. coli* were incubated in binding reaction mixtures with radiolabeled DNA fragments of the *CLP1* promoter region. Each set of four lanes in the first three panels contains 0, 1, 5, and 10 nM Sxi2**a** homeodomain protein. Lanes 1 to 4 contain a 274-bp probe (A) representing sequences farthest away from the *CLP1* gene. Lanes 5 to 8 contain a 165-bp probe (B), and lanes 9 to 12 contain a 177-bp probe (C), representing sequences closest to the *CLP1* gene. The fourth panel, lanes 13 to 15, contains probe C and increasing concentrations of the Sxi1α amino-terminal fragment, at 0, 16, and 32 nM, respectively.

development (Fig. 4A, lane 2). It was also detected readily in crosses between wild-type strains and *clp1*Δ strains (lanes 3 and 4). However, as expected, in crosses between **a** *clp1*Δ and α *clp1*Δ strains, no *CLP1* transcript was detectable (lane 5).

Because Sxi1α and Sxi2**a** are crucial regulators of the dikaryotic state, we also tested whether these proteins regulated transcription of the *CLP1* gene. RNAs from crosses between wild-type and *sxi1*αΔ and/or *sxi2***a**Δ strains under sexual development conditions were evaluated for the presence of the *CLP1* transcript. As shown in Fig. 4A, *CLP1* transcript levels in *sxi*Δ strains dropped dramatically (lanes 6 to 8), suggesting that Sxi1α and Sxi2**a** strongly upregulate the expression of *CLP1*, either directly or indirectly. It is interesting that in the *sxi*Δ strains, the wild-type *CLP1* transcript was diminished but not absent. This transcript is dependent on the presence of the *CLP1* gene, but its role is unknown.

To further characterize the *CLP1* transcript, we carried out 5′ and 3′ RACE analysis, cDNA analysis, and poly(A) tail length analysis. We found that the *CLP1* mRNA transcript is 1,686 bases long and includes both 5′ and 3′ untranslated regions (UTRs) and a poly(A) tail (Fig. 4B). Interestingly, the pre-mRNA is interrupted by two introns, one of which is in the 5′ UTR of the transcript. Introns in UTRs are rare, and although we do not know the significance of the 5′ UTR intron in *CLP1*, such sequences in other systems have been implicated in regulation of specific transcripts (8, 22). No differences in architecture were detected between the *CLP1* transcript from wild-type crosses and the *CLP1* transcript from *sxi*Δ crosses (data not shown).

***CLP1* is a direct target of Sxi1α and Sxi2a in vitro.** Inspection of the predicted promoter region of the *CLP1* gene revealed the presence of potential homeodomain binding sites ($GATGN_xACA$) approximately 211 and 510 base pairs upstream of the predicted ATG. To test the possibility that these sequences could be binding sites for Sxi1α and Sxi2**a**, we carried out EMSAs using protein fragments purified from *E. coli.* The purified proteins were tested for the ability to bind three fragments of the *CLP1* promoter. As shown in Fig. 5A, we divided the *CLP1* promoter into three fragments of 274, 165, and 177 base pairs. Each was radiolabeled and used as an EMSA probe. In the case of Sxi2**a**, a 60-amino-acid fragment containing the homeodomain region was purified and tested in EMSAs on each fragment. As shown in Fig. 5B, we detected binding to two of the three regions (lanes 5 to 12); however, binding was strong and specific only for fragment C, a 177-bp fragment located immediately upstream of the *CLP1* ORF (lanes 8 to 12). We tested the specificity of this binding by titrating competing, unlabeled fragments of either the same sequence as the probe or a nonspecific sequence into the binding reaction mixtures. Binding to fragment C was competed with only the specific competitor, indicating that binding by the Sxi2**a** homeodomain fragment occurred with high specificity (data not shown). This finding was surprising because the predicted homeodomain binding sites were located in the other two promoter fragments (A and B), and no DNA sequences with recognizable similarity to the $GATGN_xACA$ consensus were found in fragment C. For Sxi1α, we were not successful in recovering substantial quantities of active protein from *E. coli*. However, a small amount of an amino-terminal fragment of the protein (175 amino acids) containing the amino terminus through the homeodomain region was purified and tested, and it showed weak but specific binding to fragment C (Fig. 5B, lanes 13 to 15) but no binding to fragment A or B (data not shown). There was no enhancement of binding when the par-

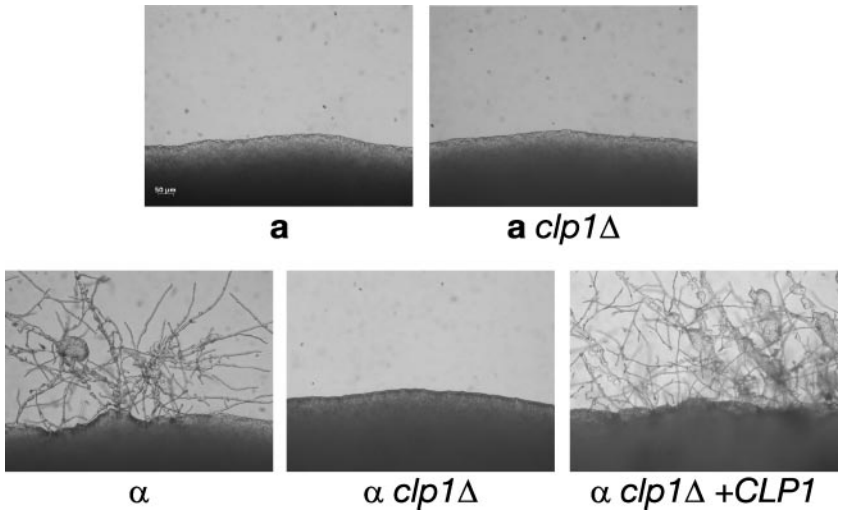

FIG. 6. *CLP1* is required for α fruiting. The top panels show wild-type **a** (JEC20) and **a** *clp1*Δ strains (CHY1069). The bottom panels show a wild-type α strain (JEC21), an α *clp1*Δ strain (CHY1072), and an α *clp1*Δ strain containing a complementing copy of the *CLP1* gene (CHY1187). All strains were photographed after 10 days at room temperature in the dark on filament agar.

tial fragments of each protein were combined with fragment C, but based on two-hybrid interaction data, we predict that larger fragments of the proteins would be required for direct protein-protein interactions (11; B. C. Stanton and C. M. Hull, unpublished data). We concluded that Sxi1α and Sxi2**a** each have the capacity to bind with specificity to fragment C. In particular, binding by the homeodomain region of Sxi2**a** is strong and specific and calls into question whether the predicted homeodomain binding sites in fragments A and B have any relevance in regulating *CLP1* or other genes in *C. neoformans*.

CLP1* is required for α fruiting.** Finally, we evaluated what role *CLP1* might play in another developmental process. In addition to the sexual development process carried out by **a** and α haploid cells, there is a developmental process carried out by α cells, known as α fruiting or monokaryotic fruiting (30). In response to severe nutrient limitation and desiccation, haploid α cells spontaneously filament and form basidia and spores in the absence of a mating partner. Interestingly, this process is independent of Sxi1α and Sxi2**a** (i.e., *sxi1*αΔ strains undergo fruiting like wild-type α strains, and **a** cells generally do not fruit), placing at least some of the early initiating signals outside the sexual development pathway controlled by Sxi1α and Sxi2**a** (12). Although we observed that *CLP1* is under the control of *SXI1*α and *SXI2a**, we also observed that *clp1*Δ strains cannot undergo α fruiting. The top panels of Fig. 6 show the responses of wild-type **a** and **a** *clp1*Δ strains to fruiting conditions. As expected, fruiting did not occur in either case, as **a** cells do not undergo α fruiting. The bottom panels of Fig. 6 show the responses of wild-type α and α *clp1*Δ strains, as well as an α *clp1*Δ strain harboring a wild-type copy of *CLP1*, to fruiting conditions. The wild-type α strain fruited as expected; however, the α *clp1*Δ strain showed no signs of filamentation. Fruiting was restored in the strain complemented with a single copy of the *CLP1* gene. This finding indicates that unlike *SXI1*α and *SXI2***a**, *CLP1* is essential for processes common to both sexual development and monokaryotic fruiting.

DISCUSSION

Sexual development is an essential part of the life cycles of most eukaryotes. In *C. neoformans* and its basidiomycete relatives, the formation of a dikaryotic filament is a key stage in this process, and it is controlled by the homeodomain transcription factors Sxi1α and Sxi2**a** (11, 18). We discovered that *CLP1*, a previously uncharacterized gene in this system, is absolutely required for sexual development, and it is the first identified direct target of Sxi1α and Sxi2**a**. The Clp1 protein, which has no identifiable predicted structural features, is not required for mate recognition or fusion with a mating partner but is critical for development after cell fusion. Cells without *CLP1* cannot progress after cell fusion, suggesting a defect in the ability to form dikaryons. However, this effect is more general than dikaryon formation alone, as we also observed defects in α fruiting and diploid formation. Although we cannot rule out that *CLP1* may play a downstream role in filament formation, it is clearly required for an early event after cell fusion.

***CLP1* and cell cycle control.** In *S. cerevisiae* and other budding yeast species, a G_1 cell cycle arrest occurs upon mate recognition, allowing the cells to prepare for fusion with a mating partner. After mating, the resulting diploids begin to grow vegetatively or undergo meiosis immediately (27, 29). In *U. maydis*, haploid cells arrest in the G_2 phase of the cell cycle in response to mating pheromone. In fact, in *U. maydis*, *CLP1* is required for dikaryotic growth in planta, and experiments suggest that Clp1 overcomes the pheromone-induced G_2 cell cycle block to allow dikaryon proliferation (23). For *C. neoformans*, little is known about the cell cycle in response to a mating partner. What is known is that in response to **a** cells, α cells form filaments (called germ tubes) in the direction of the **a** cells. The **a** cells, on the other hand, become swollen in response to α cells (13). It appears that fusion occurs between an α germ tube and the **a** partner, and it seems likely, based on

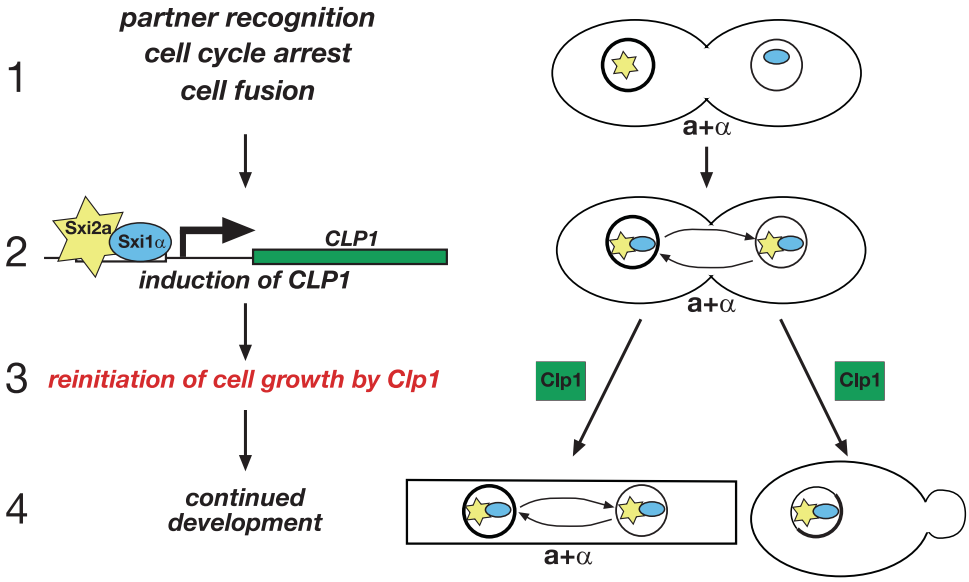

FIG. 7. Model for the role of Clp1 during sexual development in *C. neoformans*. (1) Partner recognition, cell cycle arrest, and cell fusion occur between **a** and α haploids. (2) In the resulting fusion product, Sxi1α and Sxi2**a** interact to induce expression of *CLP1*. (3) The Clp1 protein then acts to reinitiate cell growth. (4) After reinitiation, fused cells grow either as dikaryotic filaments or as diploid budding yeast cells, depending on the environmental conditions. Oval shapes represent cells. A thick circle represents the **a** nucleus, and a thin circle represents the α nucleus. The yellow star represents Sxi2**a**, the blue oval represents Sxi1α, and the green square represents Clp1.

related fungal systems, that some form of cell cycle arrest would occur to synchronize cells prior to this fusion event.

Based on our finding that *clp1*Δ cells can fuse with one another but cannot outgrow afterwards, we propose that *CLP1* in *C. neoformans* influences the cell cycle, acting as a regulator of growth reinitiation after cell fusion. Alternatively, one could argue that *CLP1* is simply required for filament formation; however, the ability to grow after fusion is also impaired during the formation of diploids (where no filaments are formed). It seems that *CLP1* in *C. neoformans* is required for escaping fusion arrest and restoring regular cell cycles, whether they be in the filament cells seen in di- and monokaryons or in the budding yeast cells formed by diploids.

***CLP1* is a target of Sxi1α and Sxi2a.** Sxi1α and Sxi2**a** are both required for sexual development, but little is known about the genes they control. It is clear from this work that *CLP1* is a target of Sxi protein regulation. *CLP1* levels were very low in haploids under all conditions tested and increased greatly during both sexual development and in diploids. For these cell types, the levels of *CLP1* transcript were greatly diminished in *sxi*Δ strains, indicating that *CLP1* is activated by the Sxi proteins. This is also true for *CLP1* in *U. maydis*, where bE and bW are regulators of *CLP1*, and the *CLP1* promoter contains the bE and bW binding site GATGN$_x$ACA (also known as a *bbs*) (21, 23). Finding such sequences in the predicted promoter region of *C. neoformans CLP1* partially motivated our study of its role in sexual development, so it is interesting that we did not detect binding to these sites by purified proteins in vitro. Instead, we observed specific binding to a region much closer to the ORF which does not contain the GATGN$_x$ACA (or a closely related) sequence. It is possible that the "consensus" sequence for fungal homeodomain binding proteins based on **a**1-α2 binding in *S. cerevisiae* and bE-bW binding in *U. maydis* does not apply as broadly as previously suspected (10, 21). Future studies with *C. neoformans* will refine the Sxi binding region in the *CLP1* promoter and reveal the sequences through which Sxi1α and Sxi2**a** regulate gene expression during sexual development.

Another interesting observation is that *CLP1* levels are simply reduced, not eliminated, in *sxi*Δ strains, suggesting that there may be low levels of Clp1 protein. *sxi*Δ strains do not have defects in cell fusion, and they can grow vegetatively after fusion; they simply do not carry out sexual development. This finding suggests that there must be a bypass mechanism for *sxi*Δ strains to continue to grow in the presence of *CLP1* that cannot be utilized in the absence of *CLP1*. It is likely that the low level of *CLP1* transcript remaining in *sxi*Δ strains is the critical factor that allows *sxi*Δ strains to escape arrest after cell fusion and to grow as budding yeast, something that *clp1*Δ strains are unable to do. This is consistent with our observation that very low levels of *CLP1* transcript are sufficient to restore wild-type levels of sexual development in mutant backgrounds (Stanton and Hull, unpublished data).

It is also possible that these lower levels of Sxi-independent *CLP1* transcript play a role in monokaryotic fruiting. Clearly, some amount of *CLP1* is required for fruiting of α strains because α *clp1*Δ strains cannot carry out this form of development; however, during fruiting, *CLP1* cannot be under the control of the Sxi proteins because there is no *SXI2***a** present, and *SXI1*α has been shown previously to be dispensable for fruiting (12). Taken together, these results indicate that during the fruiting process, *CLP1* must be under the control of a distinct pathway (independent of the Sxi proteins). The processes of monokaryotic fruiting and sexual development both require *CLP1*, but control of *CLP1* expression is managed differently between these two forms of development.

Nature of the *CLP1* transcript. To confirm the predicted structure of the *CLP1* transcript, we carried out 5′ and 3′ RACE as well as cDNA analysis and poly(A) tail length analysis. Our findings were generally consistent with our predictions, and the ORF we defined correlates with expressed sequence tag data for *CLP1* from the TIGR genome sequencing project. We did not detect additional sense transcripts like those found in *U. maydis* (23). There is, however, an expressed sequence tag in the TIGR database that corresponds to a *CLP1* antisense transcript. To determine whether this transcript might play a role in the regulation of *CLP1*, we carried out Northern assays with strand-specific probes for exon regions. Our Northern analysis consistently showed expression and regulation of the *CLP1* sense transcript. We were unable to detect the antisense transcript under any of the conditions tested, leading us to conclude that the antisense transcript is unlikely to play a role in sexual development. We cannot rule out that this RNA is significant under other conditions, leaving another pathway to explore for the role of this transcript under conditions such as those necessary for fruiting.

Summary. Using *C. neoformans* as a model of fungal sexual development provides an opportunity to understand how transcriptional regulation leads to developmental changes in simple eukaryotes. In *C. neoformans*, sexual development results in the production of spores, the likely infectious particles in human infections. The work presented here indicates that *CLP1*, a previously uncharacterized gene in this system, is essential for the development process and functions to allow developmental progression after cells of opposite mating types fuse with one another. During sexual development, *CLP1* is under the control of the transcriptional regulators Sxi1α and Sxi2**a** and is a direct target of these proteins in vitro. Taken together, these findings allow us to formulate a model in which Sxi1α and Sxi2**a** act in concert during development to upregulate the expression of the *CLP1* gene after cell fusion, allowing *CLP1* to reinitiate growth (Fig. 7). This *CLP1* function is also required for other forms of *C. neoformans* development, indicating its critical role in cell growth and, ultimately, in the production of spores.

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