A Parasitic Phase-Specific Adhesin of *Coccidioides immitis* Contributes to the Virulence of This Respiratory Fungal Pathogen

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We report the isolation of a *Coccidioides immitis* gene (SOWgp) which encodes an immunodominant, spherule outer wall glycoprotein that is presented as a component of a parasitic phase-specific, membranous layer at the cell surface. The open reading frame of the gene from *C. immitis* isolate C735 translates a 422-amino-acid (aa) polypeptide that contains 6 copies of a 41- to 47-residue tandem repeat enriched in proline (20.4 mol%) and aspartate (19.7%). Two additional isolates of *C. immitis* produce SOWgps of different molecular sizes (328 and 375 aa) and show a corresponding difference in the number of tandem repeats (four and five, respectively). The accurate molecular sizes of these proline-rich antigens, as determined by surface-enhanced laser desorption/ionization mass spectrometry, are comparable to the predicted sizes from the translated protein sequences rather than the estimated sizes based on gel-electrophoretic separation. The results of Northern hybridization confirmed that SOWgp expression is parasitic phase specific, and immunoblot studies showed that elevated levels of production of this antigen occurred during early spherule development. The recombinant polypeptide (rSOWp) was shown to bind to mammalian extracellular matrix (ECM) proteins in an in vitro assay (laminin > fibronectin > collagen type IV), suggesting that the parasitic cell surface antigen may function as an adhesin. Deletion of the SOWgp gene by using a targeted gene replacement strategy resulted in partial loss of the ability of intact spherules to bind to ECM proteins and a significant reduction in virulence of the mutant strain. The wild-type gene was restored in the mutant by homologous recombination, and the revertant strain was shown to be as virulent as the parental isolate in our murine model of coccidioidomycosis. The parasitic cell surface glycoprotein encoded by the SOWgp gene appears to function as an adhesin and contributes to the virulence of *C. immitis*.

*Coccidioides immitis* is a desert soil-inhabiting fungal pathogen that is found in the southwestern United States and in certain arid regions of Mexico, Central America, and South America (15). Air-dispersed spores (arthroconidia) produced by the saprobic phase of the fungus may be inhaled by humans who reside in or travel through these desert areas. The tiny, barrel-shaped arthroconidia (ca. 2 by 6 μm) convert into multinucleate round cells (spherules) within the lungs of the host and grow isotropically to produce large parasitic cells (60 to >100 μm in diameter). The latter undergo an elaborate process of endogenous wall growth and cytoplasmic compartmentalization, which culminates in production and the release of a multitude of endospores (each ca. 4 to 10 μm in diameter [6]). Endospores grow isotropically and differentiate into a second generation of spherules. Although young parasitic cells that are newly released from maternal spherules may be engulfed by host phagocytes, in vitro studies of host-pathogen interactions suggest that many of these fungal cells survive initial attack by the innate immune system (10). Mature spherules most likely escape phagocytosis simply because they are too large to be taken up by neutrophils and macrophages. We know from a variety of studies of coccidioidal infections in mice that the incubation period from arthroconidium inoculation to development of symptomatic disease is typically 10 to 15 days (47). It is reasonable to assume that survival of the fungus in vivo during this early period of infection and its ability to establish disease are dependent on the success of the pathogen to colonize respiratory tissue. However, no information on the mechanisms of host colonization by *C. immitis* have so far appeared in the literature.

Several lines of evidence have suggested that cell surface and secreted proteins with proline-rich regions (PRRs) play important roles in protein-protein interactions and protein folding (4, 23). These PRRs frequently occur as multiple tandem repeats and are widely distributed among prokaryotes and eukaryotes (44). Cell-surface expressed fungal and bacterial proteins which contain proline-rich tandem repeats have been suggested to function in adherence to host tissue (33, 39). An example is a glycoprotein of *Colletotrichum lindemuthianum* (CIH1) produced at the surface of intracellular hyphae which infects the tissues of bean plants. The N-terminal domain of the mature glycoprotein is rich in proline, contains several short repetitive motifs, and forms cross-links with the plant cell wall. The PRRs of CIH1 appear to be essential for establishment and maintenance of this fungus-host relationship (33). *Candida albicans* produces a glycoprotein (HWPI) at its hyphal surface that is characterized by tandemly repeated, proline- and glutamine-rich amino acid motifs (39). Unlike CIH1, it has been shown that this antigen is expressed in vivo and is developmentally regulated. The hyphal phase-specific HWPI attaches to buccal epithelial cells by an enzyme-mediated process of adhesion (40). The proline-rich motifs of HWPI are suggested to dictate a conformation of the polypeptide in which the glutamine residues are presented to and recognized by the host transglutaminase as a substrate. Digestion of the

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surface antigen apparently results in formation of cross-links between the hyphal and epithelial cell surfaces. A cell surface adhesin of *Streptococcus mutans* (P1) contains a conserved proline-rich repeat domain which is suggested to be a necessary component of conformational epitopes within the central region of the antigen (4). Deletion of this domain resulted in complete absence of the surface-localized P1 antigen. We have previously reported a major parasitic cell surface-expressed antigen produced by *C. immitis* that elicits both humoral and cellular immune responses in patients with coccidioidomycosis (20). In the present study we demonstrate that this spherule outer wall glycoprotein (SOWgp) contains tandemly repeated proline- and aspartic acid-rich motifs, functions as an adhesin, and contributes to the pathogenicity of *C. immitis* in a murine model of coccidioidomycosis.

**MATERIALS AND METHODS**

**Fungal media and growth conditions.** The *C. immitis* isolates C735, C634, and Silvera were used in this study. They were originally obtained from patients with disseminated coccidioidomycosis. The saprobiotic and parasitic phases of the fungus were grown in vitro under conditions previously described (20). Parastic-phase cultures were harvested at various times after inoculation with arthroconidia (36 to 144 h) as reported elsewhere (21).

**SOWgp gene isolation and sequence analysis.** The previously reported N-terminal and internal amino acid sequences of the purified 82-kDa SOWgp (SOWgp82 [20]) of *C. immitis* C735 were used to design degenerate sense and antisense primers. These oligonucleotide primers were used in a PCR with template genomic DNA of *C. immitis* C735 to amplify a fragment of the putative SOWgp82 gene. The nucleotide sequence of the sense primer deduced from the previously reported N-terminal amino acid sequence (GATSHKHEW) was 5'-GIGCNCNWNSCNAYAARGGARCA-3' (2,048-fold degeneracy; L, isoleucine; R is A or G; S is C or G; W is A or T; and Y is C or T). The antisense primer was designed on the basis of an amino acid sequence derived from a cyogenen bromide digestion product (KPEPPKK) (20) of the native SOWgp82 glycoprotein. The sequence of the antisense primer was 5'-YTTYTNTGGNGGYTCNG GTYT-3' (1,024-fold degeneracy). The PCR mixture (100 μl) contained 10 mM Tris-HCl (pH 8.3) plus 50 mM KCl, 1.5 mM MgCl2, final concentrations of 0.2 mM for each deoxynucleoside triphosphate and 20 μM for each primer, 50 ng of *C. immitis* genomic DNA, and 2.5 U of Taq DNA polymerase (Sigma Chemical Co., St. Louis, Mo.). Thirty-five cycles were conducted for PCR amplification in the presence of the template genomic DNA under conditions previously reported (21). The PCR products were ligated into pZErO 2.1 (Invitrogen, Carlsbad, Calif.) by the TA-cloning method (2). The clones were subsequently screened by PCR with primers derived from nucleotide sequences in the multiple cloning site of the vector. Twenty-five selected clones (inserts of 0.2 to 1.2 kb) were sequenced by using an ABI Prism 310 genetic analyzer (Perkin-Elmer, Foster City, Calif.), and the nucleotide sequences were examined by using MacDNASIS Sequence Analysis Software (version 3.5; Hitachi, San Bruno, Calif.). A 0.7-kb insert was sequenced, translated, and shown to include the reported N-terminal and internal amino acid sequences of the native SOWgp82. The 0.7-kb insert was purified, labeled with [α-32P]dCTP (~3,000 Ci/mmol; ICN, Costa Mesa, Calif.), by using a Multiprimer DNA labeling system (Amersham, Cleveland, Ohio), and employed as a probe to screen the genomic library of *C. immitis* isolate C735 (45). Positive phages were selected and amplified, and DNA was extracted for restriction enzyme digestion and Southern hybridization (46) by using the same conditions as those used for amplification of the genomic fragment. The native 82- and 60-kDa SOWgp fractions were amplified from genomic DNA (sense primer, nt 1589 to 1610; antisense primer, nt 3043 to 3200), purified, labeled with [α-32P]dCTP, and used as a probe for the Southern hybridizations.

**SOLD-TOF mass spectrometry.** The native 82- and 60-kDa SOWgp fractions of *C. immitis* isolate C735 were purified by electroelution of the protein bands from a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel after excision of the protein fractions (fractionation of the TX-114) extract of the crude spherule outer wall (SOW) (20). Surface-enhanced laser desorption/ionization-time of flight (SOLD-TOF) mass spectrometry was used to determine the molecular mass of the purified SOWgp fractions. Samples were applied to separate spots on H4 ProteinChip arrays (C-16 aliphatic chain hydrophobic surface) supplied by the manufacturer (Ciphergen Biosystems, Inc., Fremont, Calif.). To prevent cross-contamination between samples applied to the chip, a circle was drawn around each reaction spot on the chip surface by using a hydrophobic marker (Zymed Lab., Inc., San Francisco, Calif.). Each reaction spot was first activated with 2 μl of acetonitrile. Before the reactant was completely dry, a 2-μl sample (0.1 mg/ml) of the purified 82- or 60-kDa SOWgp in 0.1 M phosphate-buffered saline (PBS; pH 7.2) was applied to the reaction spot and allowed to air dry. The unbound material was removed by washing it three times with 5 μl of high-pressure liquid chromatography-grade water (Fisher Scientific, Pittsburgh, Pa.). The dried surface of each spot was finally reacted with 0.5 μl of a saturated solution which contained a patented energy absorbing molecule (EAM1; Ciphergen), which was solubilized in 50% acetonitrile plus 0.5% trihaloacetic acid in distilled water. The protein chip was then subjected to mass analysis in a model PBSII laser desorption/ionization mass spectrometer (Ciphergen). The instrument was calibrated by using purified bovine serum albumin (BSA; the molecular mass of the major fraction is 66,410 Da) on protein chips provided by the PBSII manufacturer.

**Northern hybridization.** Total RNA was isolated from 4-day mycelial cultures (saprobiotic-phase) and near-synchronous-parasitic-phase cultures at 36, 72, 96, 120, and 144 h after inoculation with arthroconidia as described previously (21).
Each RNA preparation was subjected to formaldehyde agarose gel electrophoresis as a gel norilce cultivation procedure (Hylobius abietis, Arlington, Va.) as reported elsewhere (46). The 1.1-kb SOWgp82 cDNA described above was labeled with [γ-32P]dCTP and used to hybridize with the membrane as previously described (46).

**Expression of SOWgp.** Since it was difficult to obtain sufficient amounts of the native SOWgp for antisera production and the adherence assays described below, we isolated the recombinant antigens (rSOWgp82 and rSOWgp58). The 1.1-kb SOWgp82 cDNA described above was subcloned into an EcoRI/XhoI site of the pET28b expression vector (Novagen, Madison, Wis.). Transformation of E. coli strain BL21(DE3) with the pET28b-SOWgp82 plasmid construct, induction of expression, purification, and amino acid sequence analysis of the recombinant protein were performed as previously reported (17). We also purified the 0.8-kb cDNA of SOWgp58 (Silkevich isolate) and subcloned it into pET28b for expression of rSOWgp58 by E. coli as described above. The isolation, digestion, subcloning, and expression methods were the same as described for SOWgp82.

**Production of antiserum.** BALB/c mice (6 weeks old) were used to raise antiserum against the purified rSOWgp82 by subcutaneous immunization by a standard protocol (20). The specificity of the antiserum was determined by immunoblot analysis. Preimmune mouse serum was used as a control.

**Immunoblot analysis.** Detection of the native SOWgp82 glycoprotein in total homogenates of 4-day mycelial cultures and spherical structures from selected stages of the parasitic cell cycle was conducted by SDS-PAGE separation and immunoblot analysis (21) with the above murine anti-rSOWgp82 serum. Total protein was extracted from each homogenate with 1% TX-114 (Sigma) and the concentration of protein in each sample was determined as reported earlier (20).

**Microscopy.** Freshly isolated, presegmented spherules of *C. immitis* C735 (36- to 72-h cultures; see Fig. 5) were examined by immunofluorescence microscopy using the murine anti-rSOWgp82 antibody described above. The immunofluorescence procedure was the same as that previously reported (20). In preparation for immunoelectron microscopy, in vitro-grown parasitic cells were first subjected to cryofixation by using a propane jet freezer as reported elsewhere (25).

C. The wells were then washed with PBS (0.1 M [pH 7.4]) for 12 h at 4 °C. Dickinson, Franklin Lakes, N.J.) were coated with either 50 µg/ml) as a control or different concentrations of ECM proteins (1.3 to 20 µg/ml) of the *C. immitis* isolate and revertant strains were conducted in microplates by a competitive adherence inhibition test. We chose the pAN8.1 plasmid (kindly provided by J. Woods, University of Wisconsin at Madison) containing the BLE gene which encodes a protein that confers resistance to paromomycin. We demonstrated that growth of the wild-type *C. immitis* isolate C735 was inhibited by phleomycin (Sigma) at a concentration of 1.0 µg/ml of glycosyl-extract (GYE) medium. The pAN8.1 plasmid was first digested with EcoRI and XhoI to generate a 3.4-kb fragment. The DNA fragment was then cloned into the EcoRI/XbaI site of pZErO 1.1 restriction fragment of the wild-type SOWgp82 gene. This undigested plasmid construct (pSOWgp82) was used to restore the wild-type SOWgp82 gene to the SOWgp82 mutant host by employing the protoplast method described above in the gene knockout experiment. Transformants were selected on GYE agar plates that contained phleomycin at a concentration of 4 µg/ml of growth media. Homokaryotic revertants were isolated by serial transfer of subcultures to fresh agar media containing the same antibiotic (4 µg/ml). Southern hybridization and immunoblot analyses were conducted to confirm that the selected revertant strain (SOWgp82R) contained the wild-type gene, that it integrated into the chromosomal DNA at a single site by homologous recombination, and that the level of expression of the glycoprotein was comparable to the SOWgp82 expression of the parental isolate.

**Generation of the revertant strain.** In order to select for transformants with the restored, wild-type SOWgp82 gene, we used the Δsowgp82 strain as the host and a plasmid which contained a second selection marker. We chose the pAN8.1 plasmid (kindly provided by J. Woods, University of Wisconsin at Madison) containing the BLE gene which encodes a protein that confers resistance to paromomycin. We demonstrated that growth of the wild-type *C. immitis* isolate C735 was inhibited by phleomycin (Sigma) at a concentration of 1.0 µg/ml of glycosyl-extract (GYE) medium. The pAN8.1 plasmid was first digested with EcoRI and XhoI to generate a 3.4-kb fragment. The DNA fragment was then cloned into the EcoRI/XbaI site of pZErO 1.1 restriction fragment of the wild-type SOWgp82 gene. This undigested plasmid construct (pSOWgp82) was used to restore the wild-type SOWgp82 gene to the Δsowgp82 mutant host by employing the protoplast method described above in the gene knockout experiment. Transformants were selected on GYE agar plates that contained phleomycin at a concentration of 4 µg/ml of growth media. Homokaryotic revertants were isolated by serial transfer of subcultures to fresh agar media containing the same antibiotic (4 µg/ml). Southern hybridization and immunoblot analyses were conducted to confirm that the selected revertant strain (SOWgp82R) contained the wild-type gene, that it integrated into the chromosomal DNA at a single site by homologous recombination, and that the level of expression of the glycoprotein was comparable to the SOWgp82 expression of the parental isolate.

**Expression of the phenotype, adherence, and virulence of the Δsowgp82 mutant strain.** Loss of production of the SOWgp82 glycoprotein in total cell homogenates of the mutant strain and its presence in homogenates of the parental isolate and revertant strains were confirmed by immunoblot analysis with anti-rSOWgp82 serum. Methods used for sample preparation and SDS-PAGE electrophoresis as described above. Preparation of total cell homogenates from the parental and Δsowgp82 mutant strains were also examined by immunoelectron microscopy for the presence of SOWgp82 glycoprotein by using serum from a patient with confirmed coccidioidal infection as described above.

**Evaluation of the relative adherence of intact, presegmented spherules isolated from 72-h parasitic-phase cultures of the parental and Δsowgp82 mutant strains were conducted in microplates by a competitive adherence inhibition test. Cells from the parental strain were biotinylated, streptavidin-peroxidase labeled, and washed with PBST buffer to remove the unbound label. The wells of the microtiter plate were coated with 75 µg of fibronecin or laminin/ml. The labeled spherules of the parental strain (10^9 cells in 25 µl of PBS) were mixed with an equal volume of PBS that contained different numbers of unlabeled spherules from either the parental or the mutant strain (2.5 × 10^9 to 2.0 × 10^9 cells). The mixtures of cells were added to the precoated wells, and the plates were incubated at 37°C for 2 h. The wells were washed with PBST three times and then incubated for 2 min with the peroxidase substrate. The color intensity was determined by measuring the OD_405. To control for the presence of endogenous peroxidase produced by the spherules, the cells used in this assay were first frozen at -20°C for 12 h and then tested for reactivity with the peroxidase substrate. The OD of the unlabeled cells from either the parental or mutant strain incubated with peroxidase substrate as described above was below the limit of detection by spectrophotometry. These assays were performed in triplicate, and statistical analyses were conducted by using the Student’s *t* test.

**Deletion of the SOWgp82 gene.** The pΔsowgp82 plasmid was designed as a gene replacement vector (9) and constructed by using a 3.7-kb fragment of pAN7.1 that contained the hypoxanthine-guanine-growth gene (HGP [35]). The 3.6-kb EcoRI/XhoI digestion fragment of SOWgp82 was digested with PstI to yield pΔsowgp82 construct. The latter was amplified by transformed E. coli, isolated, and used for subsequent transformation of *C. immitis* isolate C735. These procedures for transformation, the selection lines, and plasmid purification were described previously (28). The transformation procedure utilized was used to isolate homokaryotic transfectants were the same as those previously described (36). Confirmation of homologous recombination was based on results of Southern hybridization by using nucleotide probes derived from the *HGP* and targeted *C. immitis* genes. The *HGP* gene probe (i.e., probe 3 [see Fig. 7B]) was used to confirm single-site integration of the plasmid construct and consisted of a 660-bp PstI fragment generated by using the Expand high-fidelity PCR system (Amersham Pharmacia Biotech). The 0.8-kb cDNA of SOWgp82 was subcloned into an EcoRI/XhoI site of the pET28b expression vector (Novagen, Madison, Wis.). Transformation of E. coli strain BL21(DE3) with the pET28b-SOWgp82 plasmid construct, induction of expression, purification, and amino acid sequence analysis of the recombinant protein were performed as previously reported (17). We also purified the 0.8-kb cDNA of SOWgp58 (Silkevich isolate) and subcloned it into pET28b for expression of rSOWgp58 by E. coli as described above. The isolation, digestion, subcloning, and expression methods were the same as described for SOWgp82.

C. The wells were then washed with PBS, blocked with 1% BSA (for 1 h at room temperature), and reacted with 50 µl of biotin-labeled rSOWgp82 at a fixed concentration of 10 µg/ml in PBS containing 0.1% Tween 20 (PBST; Sigma) for 2 h at room temperature. The wells were washed three times with PBST, followed by incubation with 50 µl of peroxidase-conjugated streptavidin (diluted 1:1,000 in PBS; Boehringer Mannheim) for 1 h at room temperature, and then washed again as described above. Semiquantitative analysis of bound rSOWgp82 was conducted by the addition of peroxidase substrate to the wells and determination of the optical density at 450 nm (OD_450). These assays were performed in triplicate, and statistical analyses were conducted by using the Student’s *t* test.
RESULTS

Structure of the SOWgp82 gene. A 3.6-kb C. immitis clone isolated from the genomic DNA library was sequenced and shown to contain an open reading frame (ORF) of 1,266 bp. The translated sequence (422 amino acids [aa]) included the previously reported N-terminal and internal amino acid sequences of the native SOWgp82 (20). Analysis of the genomic sequence also revealed the presence of eight introns. The structure of the ORF and location of the eight introns and nine exons were confirmed by cDNA sequence analysis. The location of the N-terminal methionine (AUG) and the 5' end of the upstream UTR were resolved by using the results of the 5' RACE procedure. The 5' UTR was shown to contain a putative TATA box located 103 bp upstream of the AUG. Results of 3' RACE resolved the stop codon of the ORF and identified two poly(A) tail addition sites separated by 69 bp. An unusual feature of the nucleotide sequence of this gene is its organization into six tandem repeats. Each repeat contains a near-identical intron (54 to 56 bp long). The first and last of the six exon repeats translate 45 and 41 residues, respectively, whereas the others each translate a 47-aa sequence (Fig. 1A and B). Additional structural details of the SOWgp82 gene are provided under GenBank accession number AF308873.

Southern hybridization. Southern hybridization of electrophoretically separated chromosomal DNA suggested that the SOWgp gene is located on chromosome II (results not shown), in accordance with the nomenclature used for numbering coccidiodial chromosomes (32). Total genomic DNA preparations of C. immitis isolate C735 were separately digested with BamHI, PvuII, SalI, and XhoI, subjected to agarose gel electrophoresis (0.8 or 1.0% gels) and hybridized with the same labeled probe that was used for Southern hybridization with chromosomal DNA. Single bands were observed in each digest (5.0, 1.8, 6.0, and 3.6 kb, respectively), which were predicted on the basis of a restriction map of the genomic clone of the SOWgp82 gene. These results indicate that SOWgp82 is a single-copy gene. The same nucleotide probe and set of restriction enzymes were used for Southern hybridization with total genomic DNA digests derived from isolates C634 and Silveira (Fig. 2A and B). The results also revealed the presence of single bands for each restricted genomic fragment but showed that a clear difference exists in the size of the respective fragments from each isolate.

Protein sequence analysis and alignment. The SOWgp82 gene encodes a protein with a predicted molecular size of 46.4 kDa and a pI of 4.2. Sequence analysis performed by PSORT showed that the first 20 aa at the N terminus had the characteristic of a signal peptide, with a positively charged residue (K²), followed by a hydrophobic core and a putative cleavage site between G²⁰ and Y²¹ (Fig. 1A) (43). However, the N-terminal residue of the mature protein is G⁶¹, which was previously determined by sequence analysis of the purified, native SOWgp82 glycoprotein (20). A propeptide (aa 21 to 60; Fig. 1A) was deduced on the basis of this N-terminal sequence of the native SOWgp82 gene.
glycoprotein. The C terminus of the translated SOWgp82 contains a predicted glycosylphosphatidylinositol (GPI) signal sequence with a putative anchor site at residue G399 (http://members.nbci.com/_XMCM/jkronegg/unige/GPI-anchor/index_en.html [11]).

Examination of the amino acid composition of the deduced, full-length SOWgp82 indicated that it is a proline- and aspartate-rich protein with 16.6% proline (as mol%), 14.7% aspartic acid, 11.6% tyrosine, 10.2% glycine, and 9.2% lysine. Each amino acid repeat described above (Fig. 1A and B) is enriched in proline (20.4%), aspartate (19.7%), tyrosine (13.1%), lysine (10.9%), and glycine (10.9%). There are four cysteine residues at identical positions in each repeat, except for the fourth repeat, which contains only three cysteines (Fig. 1B). Each repeat also contains a polyproline motif (PPPPP). No N-glycosylation sites were predicted in the translated SOWgp82 sequence. However, based on positive periodic acid-Schiff (PAS) staining of the native SOWgp82 (20), we concluded that the cell surface polypeptide is linked to sugar residues. The presence of multiple serines and threonines suggest that the protein may be O glycosylated. Glycosylation of the native SOWgp82 may also be associated with the GPI anchor complex (11). The results of our BLAST searches of the GenBank and SWISS-PROT databases for homologous sequences revealed that only low similarity (<20%) exists between SOWgp82 and sequences of other reported proline-rich proteins.

Alignment of the translated SOWgp genes of three isolates of *C. immitis* showed sequence identity in the N- and C-terminal regions but differences in the number of 47-aa repeats (Fig. 3). The difference in the predicted molecular size of the SOWgps correlates with the number of conserved repeat sequences. However, the predicted molecular size of the translated, mature SOWgp (minus the signal peptide and propeptide) of each isolate and the estimated molecular size of the secreted, mature glycoprotein based on SDS-PAGE are incongruent (Table 1). The disparity is highest in SOWgp82, which contains the largest number of repeats and, therefore, the highest proline content. Proline-rich proteins typically demonstrate slow migration in SDS-PAGE gels, presumably due to conformational peculiarities that persist even after exposure to the reducing conditions of sample preparation (44).

**Molecular size of the native SOWgp determined by SELDI-TOF mass spectrometry.** SDS-PAGE gel separations of the TX-114-extracted, crude SOW fraction of strain C735 typically revealed two Coomassie blue-stained bands at 60 and 82 kDa (Fig. 4A [20]). The 60-kDa band was usually less prominent than the upper band but was clearly visible in SDS-PAGE gel separations of SOW obtained from first- and second-generation parasitic-phase cultures which contained young presegmented spherules (20). The N-terminal amino acid sequences of the purified, native SOWgp82 and SOWgp60 are identical, and it was suggested that the 60-kDa glycoprotein is a product of proteolysis of the C terminus of SOWgp82 (20). In the present study, we attempted to clarify the disparity between the molecular size of the native SOWgp fractions of isolate C735 based on SDS-PAGE and the predicted size of the mature

![FIG. 3. Amino acid sequence alignment of translated SOWgp genes of *C. immitis* isolates Silveira (SOWgp58), C634 (SOWgp66), and C735 (SOWgp82). An asterisk indicates a nonconserved substitution of residues; a filled circle indicates a conserved substitution as defined by Higgins and Sharp (18).](http://iai.asm.org/content/70/2/3447/F3)

**TABLE 1. Summary of differences between SOWgps of three clinical isolates of *C. immitis***

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Isolate</th>
<th>Sizes (kDa)* ± SD</th>
<th>No. of aa</th>
<th>No. of repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOWgp58</td>
<td>Silveira</td>
<td>(58/29.1/30.7) ± 0.3</td>
<td>328</td>
<td>4</td>
</tr>
<tr>
<td>SOWgp66</td>
<td>C634</td>
<td>(66/34.6/37.7) ± 0.3</td>
<td>375</td>
<td>5</td>
</tr>
<tr>
<td>SOWgp82</td>
<td>C735</td>
<td>(82/39.5/43.8) ± 0.3</td>
<td>422</td>
<td>6</td>
</tr>
</tbody>
</table>

*Sizes are presented as a/b/c, where a is an estimate of the molecular size of native glycoprotein based on mobility in SDS-PAGE gels under reducing conditions; b is the molecular size of SOWgp deduced from the translated gene sequence of the predicted, mature protein; and c is the molecular size of the native SOWgp as determined by SELDI-TOF mass spectrometry. The molecular sizes are mean values of three separate determinations ± the standard deviation.*
protein based on its translated gene sequence. The 60- and 82-kDa glycoproteins were each purified as previously described (Fig. 4A [20]), electroeluted from SDS-PAGE gels, and subjected to SELDI-TOF mass spectrometry to determine their actual molecular size (Fig. 4B). The results confirmed that the SDS-PAGE estimates of molecular sizes of the native, mature proline-rich proteins are markedly inflated. The actual molecular size of the secreted SOWgp82 is 43.8 kDa. This

FIG. 4. Molecular sizes of SOWgp fractions of isolate C735 as estimated by SDS-PAGE (A) and SELDI-TOF mass spectrometry (B). The asterisks in panel B locate low intensity peaks, which represent double-charged molecules.

FIG. 5. (A to C) Diagram of the parasitic cycle of C. immitis (A), Northern hybridization analysis of temporal expression of SOWgp and GAPDH genes (B), and corresponding immunoblot analysis of SOWgp82 production as detected by examination of detergent extracts of parasitic cell homogenates of isolate C735 (C). The developmental stages of first- and second-generation parasitic cells are identified by incubation times after inoculation of parasitic-phase cultures. (D and E) Immunolocalization of SOWgp in spherules (36- to 72-h stage of development) by immunofluorescence (D and E) and immunoelectron microscopy (F) with antiserum raised against the recombinant SOWp82 reveals that the antigen is presented at the parasitic cell surface. Control cells in panel E were incubated with secondary antibody-fluorescein isothiocyanate conjugate alone. IW, inner spherule wall; pm, plasma membrane. Bars: D and F, 10.0 and 0.5 μm, respectively.
value is similar to the predicted size of the mature protein (i.e., 39.5 kDa [Table 1]). SOWgp60 was shown to have an actual molecular size of 32.8 kDa, indicating that proteolysis in the C-terminal region of SOWgp82 results in the release of an ca. 10-kDa peptide. Since cleavage of this C-terminal peptide would result in loss of the GPI anchor motif, the previously reported positive PAS reaction with the mature 60-kDa fraction (20) is most likely due to putative upstream sites of O glycosylation.

Expression and immunolocalization of SOWgp82. Total RNA was isolated from saprobic- and parasitic-phase cultures of *C. immitis* isolate C735, separated by formaldehyde agarose gel electrophoresis, and hybridized with the labeled 1.1-kb cDNA of SOWgp82. Total RNA (4 μg) was extracted from mycelial cultures grown for 4 days and from each of five different developmental stages of in vitro-grown parasitic cells (36, 72, 96, 120, and 144 h after arthroconidium inoculation [Fig. 5A]). The Northern blot analysis in Fig. 5B showed that the predicted 1.6-kb cDNA band of SOWgp82 mRNA was present only in the parasitic phase. Comparison of levels of SOWgp82 gene expression in the parasitic cycle to the expression of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of *C. immitis* (Fig. 5B) (20) suggested that production of the highest amounts of the SOWgp82 transcript correlated with the early stages of isotropic growth of first- and second-generation, presegmented spherules. On the other hand, the amount of SOWgp82 mRNA sharply decreased to an almost undetectable level during the intervening stages of development.

Parallel studies of total protein were conducted by immunoblot analysis to compare levels of production of the SOWgp82 glycoprotein in TX-114 extracts of homogenates of mycelial and parasitic cells (Fig. 5C). Murine antiserum raised against the purified rSOWp82 revealed a single band in immunoblots of SDS-PAGE separations of the *E. coli* homogenates (not shown). To confirm that the recombinant protein was encoded by the SOWgp82 gene, the gel-purified rSOWp82 was subjected to Lys-C digestion and N-terminal sequence analysis (17). The amino acid sequence of a selected peptide of the digest (AA SSGSTDRPASTPV) was identical to aa 377 to 391 of the translated SOWgp82 gene (Fig. 3). In the immunoblot of different stages of *C. immitis* development (Fig. 5C), the same amount of total protein (20 μg) of each homogenate was applied to the SDS-PAGE gel as previously reported (21). The antiserum failed to recognize any component of the mycelial homogenate but recognized both the 82- and 60-kDa fractions of SOWgp82 in the parasitic cell preparations. Results of immunoblot analysis of the same developmental stages of the parasitic cycle as those described above suggested that the amount of SOWgp82 glycoprotein was highest during the early stages of spherule development in both the first and the second generations (36 to 96 h and 144 h, respectively) but decreased sharply at the time of endosporulation (120 h).

The same antiserum raised against the rSOWp82 was used for immunolocalization of the native glycoprotein in presegmented spherules by immunofluorescence (Fig. 5D and E) and immunoelectron microscopy (Fig. 5F). In both cases, the glycoprotein was found at the cell surface as patches of concentrated antigen (Fig. 5D and F). *C. immitis* spherules which were incubated with secondary antibody alone showed no label (Fig. 5E).

**Adherence of SOWgp to ECM.** The amount of recombinant SOWp produced by *E. coli* transformed with the pET-SOWgp58 plasmid construct was ~100-fold higher than the amount of bacterium-expressed rSOWp82. We cannot explain this difference in the level of expression of the two recombinant proteins. Because high sequence homology exists between rSOWp82 and rSOWp58, except for the number of 47-aa repeats (Fig. 3), we decided to use rSOWp58 for the adherence assays (Fig. 6). The results show that the labeled recombinant protein binds most avidly to laminin and significantly less so to fibronectin and collagen. A linear relationship exists between the amount of bound rSOWp58 and the concentrations of fibronectin and collagen used to test adherence. Our intent in this assay was to demonstrate that SOWgp is a putative adhesin rather than to present a detailed evaluation of the adherence properties of the cell surface molecule.

**Transformation and isolation of the Δsowgp82 strain.** The transformation plasmid construct, pΔsowgp82, was designed to integrate into *C. immitis* chromosomal DNA by homologous recombination at two crossover sites (Fig. 7A and B). Transformation of 5×10⁷ protoplasts with 5 μg of linearized DNA derived from ApaI/ClaI digestion of the plasmid construct yielded 80 to 100 hygromycin-resistant *C. immitis* colonies. A selection of 39 putative transformants were initially screened by PCR for deletion of the SOWgp82 gene fragment and integration of the plasmid into chromosomal DNA of the pathogen at a single site (36). Of the 39 colonies examined, 7 were identified as candidate, homokaryotic transformants in which the SOWgp82 gene fragment was replaced by homologous recombination with pΔsowgp82.

Three putative transformants (A7, A9, and A57) were selected from the seven colonies identified above and used for...
Southern hybridization with three different probes (probes Pb1 to Pb3; Fig. 7B). A restriction map was constructed for the 5.9-kb genomic fragment, which included the full-length SOWgp82 gene plus the 5' and 3' flanking regions (Fig. 7A). A restriction map was also constructed for the hypothetical structure of the chromosomal fragment which contained the integrated pΔsowgp82 plasmid (Fig. 7B). The results of hybridization of KpnI and BamHI digests of genomic DNA from the parental strain (P) and three selected transformants (A7, A9, and A57) with Pb1 (Fig. 7C), Pb2 (Fig. 7D), and Pb3 (Fig. 7E) provide evidence that homologous recombination occurred at a single locus in A7, A9, and A57. Pb2 and Pb3 hybridized weakly with bands in the KpnI digest but not the BamHI digest of genomic DNA of transformant A7 (Fig. 7D and E). Nevertheless, the single bands of predicted size in the Southern hybridizations of all three selected transformants indicate that...
the SOWgp82 gene fragment in these homokaryotic strains had been deleted. The A9 transformant was selected for further phenotypic analysis, and this strain was designated Δsowgp82.

Loss of SOWgp production by the Δsowgp82 transformant. Total cell homogenates of 72-h parasitic-phase cultures (see Fig. 5A) of the parental and Δsowgp82 strains were subjected to SDS-PAGE gel separation as described above and then to immunoblot analysis with the murine serum raised against rSOWp82 (Fig. 8A). The parental strain showed the characteristic 82- and 60-kDa bands of isolate C735, whereas the antiserum failed to recognize any components of the cell homogenate derived from the Δsowgp82 strain. Additional evidence that the transformant does not express the SOWgp82 glycoprotein is presented in Fig. 8B and C. Serum obtained from a patient with confirmed coccidioidal infection (36) was reacted with thin sections of the crude SOW fraction isolated from 72-h parasitic-phase cultures of the parental (Fig. 8B) and the Δsowgp82 (Fig. 8C) strains. In both cases, the electron micrographs reveal the typical membranous and amorphous material that comprises the SOW complex (7, 8). In Fig. 8B, the immunogold particles conjugated with anti-human IgG are bound primarily to the membranous components of SOW derived from the parental strain. On the other hand, no specific binding of immunogold particles to SOW of the transformant was visible (Fig. 8C).

Reduced adherence of Δsowgp82-derived presegmented spherules to ECM. Presegmented spherules isolated from 72-h cultures of the parasitic phase of the parental strain and the Δsowgp82 transformant were also tested for their ability to bind to fibronectin and laminin in a competitive adherence inhibition microplate assay (Fig. 8D). Unlabeled parental cells added to labeled parental cells at a ratio of 20:1 showed nearly complete inhibition of adherence of intact cells to the ECM proteins. Unlabeled spherules isolated from the mutant strain, on the other hand, failed to completely inhibit binding of the labeled parental cells and showed less of an inhibitory effect on binding of the parental cells to laminin than to fibronectin. These data are in agreement with the results presented in Fig. 6, which showed that purified recombinant protein (rSOWp58) binds more avidly to laminin than fibronectin. Results of this competitive inhibition assay suggest that absence of the SOWgp82 from the surface of presegmented spherules of the mutant strain significantly reduces the ability of these parasitic cells to bind to the ECM proteins.

Transformation and isolation of the SOWgp82R revertant. The transformation plasmid construct, pSOWgpR, successfully integrated into C. immitis chromosomal DNA by homologous recombination at a single crossover site, as illustrated in Fig. 9A. Transformation of 5 × 10⁷ protoplasts with 5 μg of undigested plasmid DNA yielded 15 phleomycin-resistant C. immitis colonies. The putative transformants were tested for their ability to grow in GYE agar in the presence of both hygromycin (100 μg/ml) and phleomycin (4 μg/ml). All of these colonies were screened by PCR for presence of the full-length SOWgp82 gene. The parental isolate, the Δsowgp82 mutant strain, and two selected revertants (R61 and R121) were examined by Southern hybridization (Fig. 9B) with a labeled probe (Pb2) described above (Fig. 7B). The results of hybrid-
zation of BamHI, KpnI, and XhoI digests of genomic DNA from the parental (P), mutant (A9), and two putative revertants (R61 and R121) provide evidence that the revertant strain R61 is homokaryotic and contains a single copy of the wild-type SOWgp82 gene which integrated into the chromosomal DNA by homologous recombination. The other revertant (R121) also contained the wild-type gene, but it integrated at an ectopic site. Immunoblot analysis of total protein preparations of parasitic cell homogenates from each of the four strains were conducted with antiserum raised against the recombinant SOWp82 as described above (Fig. 9C). The results show comparable amounts of SOWgp82 present in the cell homogenates of the parental (P) and revertant (R61) strains, and no protein detected by the anti-rSOWp82 antibody in the mutant strain (A9). The ectopically integrated wild-type gene in the second revertant (R121) expressed the glycoprotein but at a markedly reduced level. The revertant strain R61 was chosen for further study and was designated SOWgp82R.

FIG. 9. (A) Strategy used to restore wild-type SOWgp82 gene to the Δsowgp82 strain with a plasmid construct that contains the BLE gene which encodes resistance to phleomycin. (B) Results of Southern hybridization of restriction enzyme-digested genomic DNA of selected strains with the Pb2 probe (see Fig. 7B). Genomic DNA was obtained from the parental isolate (P), Δsowgp82 mutant (A9), and two putative revertant strains (R61 and R121). (C) SDS-PAGE separation and immunoblot analysis of total protein from parasitic cell homogenates of the same C. immitis strains as in panel B. The immunoblot was conducted with antiserum raised against the recombinant SOWp82. (D) Predicted outcome of the pSOWgpR integration event which yielded the R61 revertant strain (SOWgp82R).

Comparison of the virulence of the parental, mutant, and revertant strains. Equal numbers (n = 70) of arthroconidia isolated from the parental, Δsowgp82, and SOWgp82R strains were used to separately inoculate three groups of C57BL/10J
mice inoculated with the animals in these two groups had died by day 21. In contrast, high mortality rate beginning on day 14 postinoculation. All inoculum of the C735 parental or revertant strain showed a mice by the intranasal route (Fig. 10). Mice challenged with the mutant versus the parental strain was statistically significant (P = 0.0019).

**DISCUSSION**

We have previously described a membrane-like SOW fraction of *C. immitis* that is released into the culture medium from the surface of presegmented parasitic cells (8). This material can be easily isolated from liquid shake cultures of the parasitic phase by differential centrifugation and has been subjected to compositional analysis and immunoassays (7). Phospholipids are prominent components of this cell surface fraction (8), a compositional analysis and immunoassays (7). Phospholipids are prominent components of this cell surface fraction (8), a finding which is in agreement with the pioneering work of Tarbet and Breslau (41), who first reported that the walls of spherules are rich in “lipid complexes.” TX-114 extraction of the isolated SOW, followed by separation of the detergent and aqueous phases, yielded a water-soluble fraction composed of a single glycoprotein (SOWgp) which was detected in Coomasie blue-stained SDS-PAGE gels (20). The molecular size of this glycoprotein varies between *C. immitis* isolates. However, the N-terminal amino acid sequence of each purified SOWgp is identical, and each is reactive with sera from patients with coccidioidal infection (20). The isolated SOWgp fraction has also been shown to elicit an in vitro proliferative response of human peripheral blood mononuclear cells obtained from individuals who are skin test reactive to *C. immitis* antigen (20). Antiserum raised against SOWgp and used for immunolocalization of the antigen in mycelial and parasitic-phase preparations revealed that the glycoprotein is expressed only on the surface of presegmented spherules (20). The possibility that this immunodominant, parasitic phase-specific antigen has a significant influence on the course of coccidioidal infection prompted us to clone the SOWgp gene and explore the potential functions of the expressed glycoprotein during fungus-host interaction.

The translated SOWgp gene of isolate C735 predicted a 39.5-kDa protein composed of a signal peptide and propeptide, six tandemly repeated proline- and aspartic acid-rich motifs, and a GPI anchor signal consensus sequence. The three isolates of *C. immitis* examined here (C735, C634, and Silveira) were shown to contain six, five, and four copies, respectively, of this repeat sequence. The Southern hybridization data showed that the difference in the number of repeats is not due to alternative splicing but is instead the result of genetic polymorphism.

No N-glycosylation sites were predicted from the deduced sequence. However, earlier studies of the native SOWgp showed that it reacts with PAS stain (20). A hydrophilic profile of the deduced SOWgp protein, based on a Kyte-Doolittle plot (26; data not shown), revealed hydrophobic regions at the N and C termini that correspond to the secretory and GPI anchor signal sequences, respectively. The intervening region, composed primarily of 47-aa tandem repeats, is hydrophilic. This hydrophilic profile is characteristic of GPI-linked proteins that are anchored to the outer leaflet of the plasmalemma (11). However, immunoelectron microscopic examinations of thin sections of presegmented spherules did not show SOWgp associated with the plasma membrane. Alternatively, SOWgp could be localized to the cell surface in a manner comparable to GPI-linked mannoproteins of yeast, which have been shown to be covalently bound to cell wall polysaccharides (22). However, SOWgp was found only in association with the SOW complex at the cell surface, and our extraction procedure released the glycoprotein from the complex as a water-soluble fraction of the detergent–aqueous-phase partition (20). These data suggest that SOWgp is not covalently linked to other components of the SOW complex. However, we cannot rule out the possibility that some SOWgp remained bound to the SOW layer. Results of transmission electron microscopic studies of the crude SOW fraction have revealed that this membrane-like material has a tripartite structure in cross-section that is comparable to the ultrastructure of biomembranes (8). Isolated GPI-anchored proteins of mammalian cells have been reported to reintegrate with cell surface membranes when these proteins are added to in vitro-grown cells (29). It is possible that the SOWgp of *C. immitis* is transported in exocytotic vesicles to the spherule surface, where it integrates into the putative, amphipathic phospholipid layer of the SOW complex. The presence of cysteine residues at conserved locations in the tandem repeats of the hydrophilic domain of SOWgp (i.e., PPPPPP) have been shown to be responsible for both intramolecular and intermolecular interactions of proteins (4, 23).

The high proline content of proteins typically leads to contradictory data on molecular size based on estimates derived from the deduced protein sequence, on the one hand, and SDS-PAGE, on the other (34). Although the relatively high serine and threonine content of the C-terminal region of
SOWgp may be associated with some degree of O glycosylation, it does not account for the wide discrepancy in estimates of molecular size of the glycoprotein homologs from the three isolates examined in this study (Table 1). SELDI-TOF mass spectrometry has proved to be a valuable tool for characterization of posttranslationally modified proteins whose molecular masses cannot be accurately predicted from sequence data alone (30). The SELDI-TOF determination of molecular size of the native SOWgp of isolate C735 was 43.8 kDa, whereas the molecular size of the deduced, mature protein was 39.5 kDa. On this basis we suggest that the polypeptide is not highly glycosylated. Nevertheless, the sugar moiety may contribute to immunoreactivity and adhesion, and future studies will address these possibilities. SDS-PAGE separations of the native SOWgp from the C735 isolate consistently showed two bands with estimated molecular sizes of 82 and 60 kDa. The latter is probably a product of proteolysis, and its actual molecular size determined by SELDI-TOF analysis is 32.9 kDa. We used this same mass spectrometric method to compare the actual molecular sizes of the native, mature glycoproteins to the sequence-deduced sizes of the cloned SOWgps from C. immitis isolates C634 (37.7 versus 34.6 kDa, respectively) and Silveira (30.7 versus 29.1 kDa).

Recent studies in our laboratory have focused on C. immitis genes which demonstrate differential expression during the parasitic cycle. The rationale for these investigations is that the results may provide clues about mechanisms of morphogenetic control (21) and may simultaneously contribute to our search for potential drug targets, as well as immunoreactive and protective antigens that can be used in a vaccine against coccidioidomycosis (N. Delgado, J.-J. Yu, and G. T. Cole, Abstr. 101th Gen. Meet. Am. Soc. Microbiol., abstr. F-45, p. 363, 2001). In the present study we confirmed our earlier suggestion that SOWgp expression is parasitic phase specific. The results of both Northern hybridization and immunoblot analyses support this conclusion. SOWgp is produced during isotropic growth and early maturation of spherules but sharply decreases in expression during the endosporulation phase. Whether the decrease in specific mRNA is due to activation of a regulatory element or is the result of a precipitous loss of labile message is unknown. We recently purified a metalloproteinase (unpublished results) from the crude SOW fraction isolated from first-generation parasitic-phase cultures during the stage of endosporulation when the SOWgp content of spherules has markedly decreased compared to spherules in the isotropic growth phase (Fig. 5C). However, we have not yet determined whether the cell surface glycoprotein is specifically digested by this metalloproteinase. Immunolocalization of SOWgp with antibody raised against the recombinant protein revealed that the native glycoprotein is present only in the outer membranous and amorphous layer of the spherule wall and not in the inner wall layer. The patches of high and low density of SOWgp revealed by immunofluorescence correlated with the variable thickness of the SOW complex shown in thin sections.

Little is specifically known about the influence of SOW or SOWgp on host phagocyte interaction with parasitic cells of C. immitis. We have performed preliminary experiments (unpublished results) to test the ability of biotin-labeled recombinant SOWgp to bind to macrophages of a murine cell line (IC-21) by using a previously reported method (12). No adherence of the glycoprotein to macrophage membrane preparations was observed. An isolated, unrelated 36-kDa macrophage-binding protein of C. immitis was used as a positive control (G. T. Cole and C. Fradin, Abstr. 101th Gen. Meet. Am. Soc. Microbiol., abstr. F-13, p. 356, 2001). The results of earlier reports on interactions of polymorphonuclear neutrophils (PMNs) with parasitic cells of C. immitis suggested that the SOW complex may actually impede the ability of host cells to kill C. immitis. Frey and Drutz (13) showed that in vitro phagocytic and fungicidal activities of PMNs declined during C. immitis maturation from arthroconidia to young, presegmented spherules. These authors observed an absence of adequate PMN-spherule interaction and attributed this to production of an extracellular, fibrillar matrix that appeared to restrict intimate contact between the host and parasitic cells. This fibrillar matrix is synonymous with the SOW layer. It is possible, therefore, that the SOW complex provides protection to the pathogen against phagocytic and fungicidal activities of the host innate immune system.

Although SOWgp may not bind to the surface of macrophages, we have demonstrated here that the recombinant protein and intact, presegmented spherules adhere to host ECM proteins (laminin > fibronectin > collagen). Laminin and fibronectin are candidate ligands which could mediate adherence of the respiratory fungal pathogen to host tissues, influence colonization, and permit initiation of infection (16, 42). Host epithelial damage, which may result in exposure of the subepithelial basement membrane, is known to increase accessibility to host ligands, including laminin, fibronectin, and type IV collagen (16). In order to more critically evaluate the significance of SOWgp interaction with ECM proteins, we performed an SOWgp gene-targeted replacement experiment by using the transformation system which has recently been developed for C. immitis (36). Our previous targeted gene knock-out approach utilized a disruption protocol (36). In the present study we report the first successful gene replacement strategy (9) applied to C. immitis. The yield of transformants with confirmed homologous recombination by this method was comparable to that of the disruption procedure. The transformants showed normal mycelial and parasitic-phase growth, and presegmented spherules demonstrated characteristic release of the SOW fraction from their cell surface in liquid culture. However, the isolated and extracted SOW from the Δsowgp82 strain lacked the SOWgp component, as determined by immunoblot analysis. A surprising discovery was that SOW from the mutant was not reactive with sera from patients with confirmed, disseminated coccidioidomycosis, suggesting that the SOWgp glycoprotein is the sole component of this parasitic cell surface layer that is recognized by patient antibody. To test whether loss of the SOWgp gene product influenced adherence to ECM proteins, a competitive inhibition assay was conducted with intact, presegmented spherules. The results confirmed that the absence of the glycoprotein component of SOW results in ca. 30 to 50% reduction in the ability of parasitic cells of the mutant strain to bind to fibronectin and laminin. However, the data also suggested that additional macromolecules exist on the surface of spherules that can bind to these same ligands. Studies are under way with SOW isolated from the Δsowgp82 mutant strain to determine which additional com-
ponents of this cell surface fraction show affinity for ECM ligands.

To evaluate the effect of deletion of the SOWgp gene on host response, a comparative survival experiment was conducted in which mice were challenged intranasally with an equal inoculum of arthroconidia obtained from either the parental or ΔsowgpΔ mutant strain. A revertant strain in which the deleted SOWgp gene was restored to the disruptant was also tested in the survival experiment. This is the first report of successful generation of a revertant strain of C. immitis by homologous recombination using two separate selective markers (HPH and BLE). The results of the challenge experiment demonstrated that the mutant had significantly decreased in virulence and that the difference between the three survival curves was most evident between 16 and 21 days postchallenge. It is not known whether reduced virulence of the mutant is due to decreased efficiency of binding to host tissue, loss of a B-cell dominant surface antigen which may influence the course of immune response to the pathogen, or both. Parasitic cell surface adhesins isolated from Blastomyces dermatitidis (BAD1 [5]) and Paracoccidioides brasiliensis (gp43 [3]) have also been reported to elicit both strong humoral and cellular immune responses during the course of the respective mycoses. As in coccidioidal infection, activation of the T helper 1 (Th1) rather than the Th2 subset of T cells appears to be essential for immunoprotection against these fungal diseases (20). All three antigens (BAD1, gp43, and SOWgp) contain immunodominant B-cell epitopes (24, 37). High titers of antibody to gp43 in infected mice with P. brasiliensis were correlated with T-cell hypersensitivity (3). These authors suggested that patient Th2 response to the immunodominant gp43 antigen may have compromised the ability of the host to control P. brasiliensis infection. SOWgp is a structurally complex antigen associated with a still undefined cell surface matrix (SOW). We suggest that SOWgp and other components of the SOW layer may play important roles both in adherence to host tissue and modulation of the immune response to C. immitis infection.

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