Molecular Cloning and Characterization of *Coccidioides immitis* Antigen 2 cDNA

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Previous experiments have provided evidence that *Coccidioides immitis* antigen 2 (Ag2) is a major T-cell-reactive component of mycelia and spherule cell walls. Here we report the identification and cloning of the cDNA that encodes Ag2 from a lambda ZAP cDNA expression library constructed from spherule-derived RNA. DNA sequence analysis established that the 1,255-bp clone contains a 174-bp 5' untranslated region, a 582-bp open reading frame which encodes for a protein consisting of 194 amino acids, and a 375-bp 3' untranslated region, including a poly(A) tail. The recombinant Ag2 protein has a predicted molecular mass of 19.5 kDa and contains an 18-amino-acid N terminus which has been tentatively identified as a signal peptide. The Ag2 cDNA was ligated into the pGEX-4T-3 vector and expressed in *Escherichia coli* TG-1 cells as a glutathione S-transferase fusion protein. The recombinant fusion protein showed reactivity with sera from patients with coccidioidomycosis and elicited delayed-type footpad hypersensitivity responses in *Coccidioides*-immune mice. These results suggest that the Ag2 cDNA can be used for the large-scale production of this immunologically important protein.

Coccidioides immitis, the etiologic agent of coccidioidomycosis, is a dimorphic fungus which exists in the soils of arid and semiarid regions of the Western Hemisphere (12, 14). This fungus propagates in a mycelial form that produces infectious arthroconidia which become airborne. When inhaled by a susceptible host, arthroconidia undergo a morphological conversion into endospore-forming spherules. Although 60% of primary infections are asymptomatic, approximately 5% of primary infections develop into a persistent pulmonary disease and 1% of infections progress into a disseminated disease, commonly involving the skin, bone and joint tissues, and the central nervous system. Resistance to the disease is associated with the acquisition of a delayed-type hypersensitivity and production of the T-helper 1 cytokines, gamma interferon and interleukin-2 (1, 4, 19).

Previous studies from this laboratory showed that an alkali-soluble, water-soluble cell wall extract, designated C-ASWS, elicited T-cell responses in patients with coccidioidomycosis and in experimentally infected animals and protected mice against lethal challenge with *C. immitis* (8, 11, 18, 24). Immunochemical analyses established that the C-ASWS extract contains two components, a polymeric glycosylated protein, designated antigen 2 (Ag2), and a polysaccharide antigen that reacts with anti-*Coccidioides* immunoglobulin M (IgM) tube precipitin (TP) antibody (3, 5, 6). These studies, together with the consistent demonstration that Ag2 is present in extracts that elicit T-cell responses (3, 4), provide evidence that Ag2 is a major T-cell-reactive component of *C. immitis*. However, attempts to purify Ag2 from the TP polysaccharide by physicochemical procedures or immunoaffinity chromatography have been unsuccessful, a result that suggests these two antigens are physically or chemically complexed (4, 5). Using anhydrous hydrogen fluoride to deglycosylate a spherule-phase lysate, Dugger et al. (13) isolated a 33-kDa peptide which, by tandem two-dimensional immunoelectrophoresis, showed a reaction of identity with the anodal peak of Ag2. Although their

procedure yielded an immunoreactive peptide (13, 15), the multiple steps required to achieve purification make it impractical for use in large-scale production of this antigen. We therefore sought to utilize the techniques of molecular biology to identify and clone the cDNA that encodes Ag2.

(A preliminary report of this work has been presented previously [26]).

MATERIALS AND METHODS

Antisera. Goat anti-coccidioidin (CDN) serum, goat antiserum specific to the Ag2-TP complex, and the murine IgG2a monoclonal antibody (MAb) to Ag2 were previously described (5, 6, 9). Normal goat sera and an irrelevant murine IgG1 MAb were included as negative controls. Serum samples were also obtained from patients with coccidioidomycosis and, for a negative control, from healthy, CDN skin test-negative persons. All antibodies, except human sera, were preadsorbed with an *Escherichia coli*-phage lysate (Stratagene, La Jolla, Calif.) before use.

Extraction of total RNA. Spherule cultures were prepared by inoculating arthroconidia of *C. immitis* (Silveira; ATCC 28868) into a modified Converse broth medium as described previously (7) and incubating cells at 40°C on a gyratory shaker (200 rpm). Once the culture medium became turbid, two-thirds of the volume was removed and fresh medium was added. Spherules were harvested by centrifugation at 450 × g for 15 min and washed once in diethyl pyrocarbonate-treated distilled water. Spherules were mechanically disrupted by mixing 1 volume of cells, suspended in diethyl pyrocarbonate-water, with 5 volumes of glass beads (diameter, 0.45 to 0.60 mm; Sigma Chemical Co., St. Louis, Mo.) and shaking the suspension in a Braun homogenizer for 90 s at 2,000 rpm. A CO₂ cooling device was used before and during breakage to minimize heat effects. The homogenate was diluted in TriSolv reagent (BIOTECX Laboratories, Inc., Houston, Tex.) to completely lyse cells. RNA was extracted by adding chloroform, and after the mixture was shaken vigorously, the RNA was precipitated from the aqueous phase with isopropanol.

Construction and immunoscreening. Isolation of poly(A)⁺ RNA, synthesis of double-stranded cDNA, and construction of the cDNA expression library were performed with a Poly(A) Quick mRNA isolation kit, ZAP-Express cDNA synthesis kit, and ZAP-Express cDNA GigapackII Gold cloning kit, respectively, from Stratagene. Briefly, mRNA was purified from 4 mg of total RNA by using a poly(dT)-nitrocellulose column. The purified mRNA (4 µg) was reverse transcribed to first-strand cDNA in the presence of StrataScript RNase H⁻ reverse transcriptase and oligo(dT) primer, with subsequent second-strand cDNA synthesis using *E. coli* DNA polymerase I and poly(A) primer (Stratagene). Then double-stranded cDNA was blunt ended and ligated to adapters containing *Eco*RI and *Xho*I sites. The cDNA fragments bearing *Eco*RI and *Xho*I sites were ligated into ZAP Express vector, pre-cut with the same restriction enzymes, to obtain a unidirectional recombinant phage library. The titers of recombinant phage were determined by using *E. coli* XL1-Blue MRF' cells (Stratagene) as host cells and plating on NZY (GIBCO BRL Lifescience) plates with 0.7% top

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agarose premixed with isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). Recombinant phage were enumerated after being incubated at 37°C for 6 to 8 h. The cDNA expression library was amplified once before use.

Approximately 10^4 amplified recombinant phage and *E. coli* XL1-Blue MRF⁺ cells were coinoculated at 37°C for 15 min and then plated on NZY agar plates. After a 3-h incubation at 42°C, protein expression was induced by placing a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) presoaked in IPTG (10 mM) directly onto the agar. Plates were incubated at 37°C for 4 h, at which time the membrane was removed and washed in blocking buffer. Primary immunoscreening was performed with a polyclonal goat anti-CDN serum. Thirty-five positive clones from primary screening were combined for secondary screening with hyperimmune goat anti-Ag2-TP serum and murine anti-Ag2 MAb. Goat antisera were diluted 1:2,000 in Tris-buffered saline (20 mM Tris, 0.15 M NaCl, pH 7.4) containing 0.05% Tween 20 and 1% bovine serum albumin (BSA); the MAb was diluted 1:100 in the same buffer, except that the pH of the Tris-buffered saline was 8.6, which is optimal for its reactivity with Ag2 (9). After a 90-min incubation at room temperature, alkaline phosphatase-conjugated rabbit anti-goat IgG or goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added at a 1:1,000 dilution. The reactions were developed by the addition of 5-bromo-4-chloro-3-indolylphosphate (BCIP)-Nitro Blue Tetrazolium (Sigma Chemical Co.). A total of 19 positive clones (13 detected with goat anti-Ag2-TP serum and 6 detected with the anti-Ag2 MAb) were isolated, replated, and plaque purified three times by using the same antibodies.

DNA sequencing. The *C. immitis* cDNA inserts from the 19 purified clones were individually subcloned into pBK-CMV vector (Stratagene) by in vivo excision using the R408 helper phage (Stratagene). Recombinant plasmids were purified by using a Wizard miniprep system (Promega Corporation, Madison, Wis.) and then partially sequenced from both the 3' and 5' ends by the ³⁵S-dATP dideoxy chain termination method (21) using Sequenase version 2.0 with T3 (5'-ATTAACCTCACTAAAG-3') and T7 (5'-TAATACGACTCACTATAGGG) primers (United States Biochemical, Cleveland, Ohio). On the basis of sequence analyses, the cDNA insert from a representative clone, designated A3, was fully sequenced at the Center for Advanced DNA Technologies at the University of Texas Health Science Center in San Antonio with an Applied Biosystems (Foster City, Calif.) 373A DNA Sequencer.

PCR cloning of 5' fragment. The 5' Ag2 cDNA fragment that was absent from the clones obtained by immunoscreening was generated by PCR cloning. Oligonucleotides 5'-CTATGACCTTGATTACGC-3' (from pBK-CMV) and 5'-GAT GTTGAGACGGAGAT-3' (from known Ag2 cDNA sequence) were designed to amplify the unknown 5' region. The 5' Ag2 cDNA fragment was amplified from 2×10^6 recombinant phage according to the manufacturer's (Boehringer Mannheim, Indianapolis, Ind.) protocol and cloned into the pGEM-T vector (Promega).

Construction of plasmids. Four plasmids were constructed. (i) pBK-CMV-A3 was generated by subcloning a 1,035-bp *EcoRI-XhoI* fragment from lambda ZAP recombinant phage A3 (Stratagene) into pBK-CMV vector, (ii) pGEX-4T-3-A3 fusion plasmid was generated by subcloning an in-frame *EcoRI-XhoI* Ag2 cDNA fragment from pBK-CMV-A3 into *EcoRI-XhoI*-precut pGEX-4T-3 vector (Pharmacia, Piscataway, N.J.) at a site downstream from the glutathione S-transferase (GST) gene, (iii) pGEM-T-A3 was generated by cloning a PCR-amplified internal Ag2 cDNA fragment into pGEM-T vector with 5'-ACTCGCTTGACCGACTTCAA-3' and 5'-ACAGGATAAGTAATAAGTG TGA-3' as primers, and (iv) pGEM-T-A3-5' was generated by cloning a PCR-amplified 5' Ag2 cDNA fragment into pGEM-T vector.

Northern (RNA) analysis. RNA from spherules (40 μ g) was electrophoresed in a 1.2% agarose-formaldehyde gel at 5 V/cm for 5 to 6 h and then transferred onto a Nytran membrane (Schleicher & Schuell) by capillary blotting. Hybridization and colorimetric development were performed with a Genius I nonradioactive random primer labeling system (Boehringer Mannheim). Briefly, the membrane was hybridized with a digoxigenin-labeled 875-bp Ag2 cDNA probe that was released by cutting pGEM-T-A3 with *NorI* and *ApaI*. The membrane was washed twice with $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) at 25°C and then with $0.2 \times$ SSC-0.1% SDS at 68°C. The membrane was blocked in TBST-BSA (Tris-buffered saline containing 0.05% Tween 20 and 3% BSA), and the reactions were visualized after incubation with 1:5,000 alkaline phosphatase-conjugated goat anti-digoxigenin IgG and then addition of BCIP-Nitro Blue tetrazolium.

Expression and purification of GST-Ag2 fusion protein. The pGEX-4T-3-A3 fusion plasmid was transformed into *E. coli* TG-1 cells for expression of the GST-Ag2 fusion protein (22). As a control for the GST peptide alone, *E. coli* host cells were also transformed with the parental pGEX-4T-3 plasmid. pGEX-4T-3-A3- and pGEX-4T-3-transformed cells were grown in 400 ml of $2 \times$ YTA medium (1% yeast extract, 1.6% tryptone, and 100 μ g of ampicillin per ml in 0.9% NaCl, pH 7.0) at 37°C in a shaking incubator until the culture medium showed an optical density of 0.6 to 0.8 at 600 nm. IPTG was added to a final concentration of 0.5 mM, and after a 3-h induction period (which was shown to be optimal in preliminary experiments), cells were harvested by centrifugation and washed once in 60 ml of ice-cold phosphate-buffered saline (PBS; pH 7.2).

The pellets were lysed by three successive freeze-thaw cycles of 3 min in liquid nitrogen followed by 7 min in ice water (17). The cell pellets were resuspended in 5 ml of ice-cold PBS, and after centrifugation, the soluble lysate fraction was

collected and mixed with 200 μ l of glutathione-Sepharose 4B beads (Pharmacia). The mixtures were incubated at room temperature with periodic shaking, and after three washes in ice-cold PBS, the affinity-purified GST-Ag2 fusion protein and GST peptide control were eluted from beads by the addition of 2 ml of reduced glutathione. The eluates were stored at -80°C until assayed.

SDS-PAGE and Western blot (immunoblot) analysis. Approximately 200 ng each of affinity-purified GST-Ag2 fusion protein and the GST peptide alone was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in 4 to 20% gradient polyacrylamide gels. Molecular size markers (GIBCO and Novel Experimental Technology, San Diego, Calif.) were electrophoresed in parallel with samples. After electrophoresis, gels were either stained for protein with Coomassie blue or assayed for reactivity with anti-*Coccidioides* antibodies by Western blotting. For the latter, SDS-PAGE-separated proteins were electroblotted onto nitrocellulose membranes and, after the membranes were blocked with TBST-BSA, goat anti-Ag2-TP, normal goat serum, sera from patients with coccidioidomycosis, and serum from a healthy subject were added to individual strips. Goat sera were used at a dilution of 1:1,000; human sera were diluted 1:200. The reactions were developed by the addition of alkaline phosphatase-conjugated rabbit anti-goat or goat anti-human IgG (Kirkegaard and Perry Laboratories), depending upon the primary antibody, followed by the addition of BCIP-Nitro Blue Tetrazolium substrate.

Enzyme-linked immunosorbent assay (ELISA). A 96-well plate was coated with recombinant GST-Ag2 at the concentration of 500 ng per well in a carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C and blocked with TBST-BSA. The wells were incubated with 10-fold serial dilutions of the anti-Ag2 MAb or an irrelevant murine MAb for 1 h at 37°C. Alkaline phosphatase-labeled goat anti-mouse antibody was added, and after a 1-h incubation, *p*-nitrophenylphosphate diluted in 1 M diethanolamine-0.5 mM MgCl₂ buffer (pH 9.8) was added. The reactions were read at A_{405} .

Footpad testing. The procedures for immunizing and footpad testing of mice have been detailed previously (10). In brief, DBA/2 mice (Charles River Laboratories, Charleston, S.C.) were immunized with a total of 2.1 mg (dry weight) of formalin-killed spherules (FKS) of *C. immitis* Silveira administered in three weekly intramuscular injections (18). Eight weeks after the last injection, five FKS-immunized mice and, for comparison, five nonimmunized mice were footpad tested for delayed-type hypersensitivity by injecting the right or left hind footpads with either GST-Ag2 or GST alone, each at a protein concentration of 3 μ g. Separate groups of immunized and nonimmunized mice were footpad tested with C-ASWS (10 μ g [dry weight]) or saline alone in the right and left hind footpads. Just before and 24 h after injection, footpad thicknesses were measured with a dial caliper (Mitutoyo, Tokyo, Japan). The results were calculated as the difference in footpad thickness of antigen- and saline-injected pads at 24 h minus the difference in footpad thickness of antigen- and saline-injected pads before footpad challenge. The differences in the responses elicited in immunized and nonimmunized mice were evaluated for statistical significance by Student's unpaired *t* test.

Nucleotide sequence accession number. The nucleotide sequence and deduced amino acid sequence of the cloned Ag2 cDNA have been submitted to the GenBank database under accession no. U32518.

RESULTS

Cloning and sequencing of Ag2 cDNA. Among 35 positive clones selected by primary screening, 19 clones showed reactivity with both the polyclonal goat anti-Ag2-TP serum and the anti-Ag2 MAb. Preliminary nucleotide sequence analyses at both the 3' and 5' ends indicated that the cDNA inserts from 15 of these 19 clones were identical. One of these, clone A3, was chosen as the representative clone and fully sequenced. This 1,255-bp cDNA contains a 582-bp open reading frame and a 375-bp 3' untranslated region, including a poly(A) tail, but lacks a 5' start site. To obtain the full-length open reading frame, the 5' region was cloned directly from the lambda ZAP cDNA expression library by PCR using an antisense primer from an internal cDNA sequence and a sense primer from the pBK-CMV vector sequence. The 583-bp PCR-generated fragment was cloned into the pGEM-T vector and sequenced. The results (Fig. 1) established that the cloned Ag2 cDNA contains a 174-bp untranslated 5' terminus, an open reading frame that encodes a 194-amino-acid protein, and a 468-bp untranslated 3' end, including a 22-bp poly(A) tail.

Northern analysis. The size of the Ag2 mRNA transcript was determined by Northern blot analysis. Hybridization of spherule RNA with a digoxigenin-labeled probe that contained a 875-bp fragment of Ag2 cDNA yielded a single band with an

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1  ctctccctcccccttagtccctcagttattttcttccccatataaaacatacattcgt
61  ttctgtgttccatcaaaagactatcgttaattctccaaacccggttctgttttttttctg
121 tactattaggaggataatcgttctctcgttagacgcacatacataatcaaaatgcag
      M Q 2
181  ttctctcagctctcctcgtctctcgtctcgtcgcggcctcgcagtgccagctccagac
      F S H A L I A L V A A G L A S A Q L P D 22
241  atccacaccttgcctctcaactgctctcgttggagctctcggcaacgatggctgcacccg
      I P P C A L N C F V E A L G N D G C T R 42
301  ttgaccgacttcaagtgcacactgctccaagcctgagctccaggacagatcactccttgc
      L T D F K C H C S K P E L P G Q I T P C 62
361  gttgaggaggtcctcctctcgcacgcccgttatctcgtctccaaacatcgtctgtgaccag
      V E E A C P L D A R I S V S N I V V D Q 82
421  tgcctcaaggccggttcccaattgacatccaccagttgacaccacccgctcccccag
      C S K A G V P I D I P P V D T T A A P E 102
481  ccattccgagacccgctgagccacccgctgagccacccgagggagccacccgctccacc
      P S E T A E P T A E P T E E P T A E P T 122
541  gctgagccacccgctgagccgactcatgagccacccgagggagccacccgctcccaacc
      A E P T A E P T H E P T E E P T A V P T 142
601  ggcactggcggtggttccccactggcaccggttctctcaccgtcactggcagaccact
      G T G G G V P T G T G S F T V T G R P T 162
661  gccctccacccagctgagttccacggtgctggctccaaacgctcgtgacagctgtggcgcc
      A S T P A E P P G A G S N V R A S V G G 182
721  attgctgctgctctcctcgtctcgtctcctacctgtataatttagactatcagcaaaact
      I A A L L G L A A Y L * 194
781  gacaagcagctgcccattggcgtcaattatcccccccgccatttttccagttcattttt
841  ttctcgacaacaattcagcagcattggaagcgcaaatgacccggatgctcgcgaacacga
901  taaaaggttttcaagatctctctctctcgtctcctcctctctggaggctctaatgttcat
961  ttggatgaaatggggcgatgggcaatcgttctgtgggggtgaaggagtgaggatctgg
1021 gatcgggatcccgaaagtcacaaacgcgtttattctcgcgagtgtgaccacggctatgggt
1081 gtacaatgttgtctgtgactttttacggggggggagaaacgaataaacacacctga
1141 ttctacttccacatttatttacttactctatctaatgtacatctgggaaggagcttat
1201 ttccctgttaataatttggtgttatatttgttataaaaaaataaaaaa

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FIG. 1. Nucleotide sequence and deduced amino acid sequence of cloned Ag2 cDNA. DNA base numbers are on the left, and amino acid numbers are on the right. The underlined amino acid region at the beginning of the N terminus of the Ag2 protein is the proposed signal peptide. The amino acid sequence region in bold contains the tetrapeptide repeats. The underlined regions from nucleotides 391 to 408 and from nucleotides 1148 to 1169 contain the sequences for the primer set used to amplify the cDNA fragment employed as the probe in Northern blot assays. The underlined region from nucleotides 295 to 316 contains the sequence for the antisense primer used to clone the 5' end of the Ag2 cDNA. The stop codon is indicated by the asterisk.

approximate molecular size of 1.3 kb (Fig. 2), consistent with that of the cloned cDNA.

Expression and immunoreactivity of the recombinant GST-Ag2 product. The Ag2 cDNA was inserted into the pGEX-4T-3 vector and expressed in *E. coli* TG-1 cells. After 3 to 4 h of induction with IPTG, host cells were lysed and the GST-Ag2 fusion protein was purified by affinity chromatography on glutathione-Sepharose 4B beads.

Analysis of the purified GST-Ag2 protein by SDS-PAGE showed two bands with estimated molecular masses of 57 and 52 kDa (Fig. 3A). Both bands were recognized in Western blots probed with goat anti-Ag2-TP serum and serum from a patient with coccidioidomycosis but not normal goat serum or serum from a healthy human subject (Fig. 3B). The 57- and 52-kDa bands were also recognized by goat anti-GST antibody

FIG. 2. Northern blot analysis of spherule-phase RNA. Total cellular RNA (40 µg) was electrophoresed in a 1.2% agarose gel, transferred onto a Nytran membrane, and hybridized with a digoxigenin-labeled oligonucleotide probe for an 875-bp fragment of Ag2 cDNA as described in Materials and Methods. The position of the single 1.3-kb band is shown on the left.

FIG. 3. (A) SDS-PAGE analysis showing the GST-Ag2 recombinant product expressed as two peptides with molecular masses of 57 and 52 kDa. The 29-kDa band represents dissociated GST peptide. (B) Western blot analysis showing the 57- and 52-kDa peptides recognized by goat anti-Ag2-TP serum and serum from a patient with coccidioidomycosis but not by normal goat serum or serum from a healthy, CDN skin test-negative subject (normal).

(results not shown), and since GST has a molecular mass of 29 kDa, the recombinant Ag2 moieties have masses of 28 and 23 kDa, respectively.

Since an earlier study had shown that the anti-Ag2 MAb is directed against a conformational epitope (9), as judged by the lack of demonstrable activity in immunoblots with antigen electrophoresed under denaturing conditions, the reactivity of this MAb with the recombinant Ag2 was evaluated by ELISA. The results (Fig. 4) established that the recombinant GST-Ag2 fusion protein was selectively recognized by the anti-Ag2 MAb.

The capacity of the recombinant protein to elicit a cell-mediated immune response was evaluated in footpad tests of FKS-immunized DBA/2 mice. As shown in Table 1, the GST-Ag2 fusion protein elicited a mean footpad response of $(27.9 \pm 6.0) \times 10^{-2}$ mm in FKS-immunized mice versus $(8.1 \pm 1.5) \times 10^{-2}$ mm in nonimmunized controls ($P < 0.001$ by Student's unpaired *t* test). Footpad responses to GST alone were essentially negative. For comparison, a separate group of mice was footpad tested with the Ag2-enriched C-ASWS cell wall extract. The mean footpad response obtained in the FKS-immunized group was $(30.5 \pm 8.3) \times 10^{-2}$ mm, a result comparable to that obtained with the recombinant Ag2 protein ($P > 0.05$).

Analyses of predicted Ag2 amino acid sequences. Previous investigations have shown that Ag2 is located in cell walls (3, 15), and on this basis, we predicted that this antigen contains a signal peptide. Consistent with this prediction, the N terminus of the translated Ag2 cDNA contains a positively charged His-5, followed by a series of hydrophobic amino acids and a putative signal peptide cleavage site between Ala-18 and Gln-19 (20, 23, 25). The deduced amino acid sequence of Ag2 also shows a region (from residues 98 through 141) that contains 10 repeats of the tetrapeptide TXX'P, where X is alanine, histidine, or glutamic acid and X' is alanine, valine, or glutamic acid (Fig. 1). A GenBank database search revealed partial sequence homology between the Ag2 tetrapeptide repeat region and a TGTP tandem repeat region in *Dictyostelium discoideum*. Of potential relevance, the gene encoding the TGTP-containing protein in *D. discoideum* is developmentally regulated and expressed during spore germination (16).

FIG. 4. Reactivity of anti-Ag2 MAb with the GST-Ag2 recombinant, as assessed by ELISA. Anti-Ag2 MAb and, for a comparative control, an irrelevant MAb were reacted with the GST-Ag2 fusion protein (500 ng per well), and after the removal of nonreactive antibodies, alkaline phosphatase-conjugated goat anti-mouse IgG, followed by substrate, was added. The results were read at A_{405} .

DISCUSSION

Previous investigations have suggested but not proven that Ag2 is a T-cell-reactive component of *C. immitis* cell walls (3, 8, 11, 24). Despite numerous attempts by us and others to purify Ag2 to homogeneity, the resulting preparations have invariably contained detectable levels of the TP polysaccharide antigen. Since this problem could best be resolved by cloning and expressing the gene that encodes Ag2, we constructed a *Coccidioides* cDNA lambda ZAP expression library and, by using anti-Ag2 antibodies for the detection of Ag2 recombinants, identified and cloned Ag2 cDNA. Nucleic acid sequencing showed that the cloned 1,255-bp cDNA contains an open reading frame of 582 bp which encodes a 194-amino-acid protein with an 18-amino-acid N-terminal signal peptide. Expression of this cDNA using the pGEX-4T-3 vector yields a GST-Ag2 fusion protein which shows reactivity with anti-*Coccidioides* polyclonal antibodies and MAbs and elicits delayed-type footpad hypersensitivity responses in *Coccidioides*-immune mice.

Analysis of the affinity-purified GST-Ag2 fusion protein by SDS-PAGE and immunoblotting revealed two bands with approximate molecular masses of 57 and 52 kDa, both of which

reacted with anti-Ag2 and anti-GST antibodies in Western blots. We do not have a definitive explanation at this time as to why the Ag2 cDNA clone expresses two bands as opposed to one. Considering that the cDNA clone was plaque purified three times, we believe that it is highly unlikely that two or more clones are expressed. Alternative explanations include either proteolytic degradation during isolation of the recombinant protein or posttranslational modification. Although we cannot exclude proteolysis during the synthesis of the recombinant in *E. coli* host cells, degradation during purification of the recombinant product seems remote since protease inhibitors did not prevent the detection of two bands in soluble lysates from host cells. The possibility that the recombinant protein undergoes posttranslational modification in procaryotic host cells that differs from that in eucaryotic cells has not been ruled out and is being explored by expressing the Ag2 cDNA in the eucaryotic baculovirus system.

The cloned Ag2 cDNA contains a region with 10 repeats of the tetrapeptide TXX'P. A gene encoding a protein with a somewhat similar tetrapeptide repeat (TGTP) has been shown to be differentially regulated during spore germination in *D. discoideum* (16) and is believed to function as a cellulase (2, 16). The possibility that the Ag2 tetrapeptide repeat is a spore-specific peptide seems unlikely since Ag2 is demonstrable in cell walls and cell lysates of mycelia and spherules of *C. immitis* (3, 7). On the other hand, if the family of spore-germinating-specific proteins of *D. discoideum* function as cellulolytic enzymes (2, 16), one could envision a similar spore-germinating protein in the formation of endospores as well as enterothallic arthroconidia. It would be of fundamental interest to examine the differential expression of Ag2 mRNA and protein during the morphogenetic phases of *C. immitis*.

A major goal of many workers in mycology has been to obtain sufficient quantities of highly purified antigens for use in the development of immunodiagnostic assays. The isolation and cloning of the cDNA that encodes Ag2 will enable large-scale production and evaluation of Ag2 as an immunodiagnostic antigen in cellular and humoral immune assays and as a vaccine against *C. immitis*. The cloned Ag2 cDNA will also provide a useful probe for examining in vivo expression of this protein during the morphogenesis of *C. immitis* saprobic- and parasitic-phase cells and its possible role as a structural and regulatory protein.

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ADDENDUM

Since the initial review of this manuscript, Dugger et al. have published a brief communication on the cloning and sequence analysis of a cDNA that encodes a putative immunoreactive protein (13a). The nucleotide and deduced amino acid sequences of their cloned cDNA are concordant with those of our Ag2 cDNA.

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TABLE 1. Induction of delayed-type footpad hypersensitivity responses by recombinant Ag2 in FKS-immunized mice^a

Footpad injection of:	Mean footpad response (10 ⁻² mm)	
	FKS-immunized mice	Nonimmunized mice
GST-Ag2	27.9 ± 6.0 ^b	8.1 ± 1.5
GST	4.1 ± 2.2	6.6 ± 1.7
C-ASWS	30.5 ± 8.3 ^b	7.1 ± 1.9
Tris-buffered saline	0	1.0 ± 0.6

^a Data are the means ± standard errors for groups of five mice.

^b Significantly increased when compared with the response of nonimmunized mice ($P < 0.001$) by Student's unpaired *t* test.

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