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Molecular Cloning of cDNA and Analysis of Protein Secondary Structure of *Candida albicans* Enolase, an Abundant, Immunodominant Glycolytic Enzyme

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We isolated and sequenced a clone for *Candida albicans* enolase from a *C. albicans* cDNA library by using molecular genetic techniques. The 1.4-kbp cDNA encoded one long open reading frame of 440 amino acids which was 87 and 75% similar to predicted enolases of *Saccharomyces cerevisiae* and enolases from other organisms, respectively. The cDNA included the entire coding region and predicted a protein of molecular weight 47,178. The codon usage was highly biased and similar to that found for the highly expressed EF-1α proteins of *C. albicans*. Northern (RNA) blot analysis showed that the enolase cDNA hybridized to an abundant *C. albicans* mRNA of 1.5 kb present in both yeast and hyphal growth forms. The polypeptide product of the cloned cDNA, which was purified as a recombinant protein fused to glutathione *S*-transferase, had enolase enzymatic activity and inhibited radioimmunoprecipitation of a single *C. albicans* protein of molecular weight 47,000. Analysis of the predicted *C. albicans* enolase showed strong conservation in regions of α helices, β sheets, and β turns, as determined by comparison with the crystal structure of apo-enolase A of *S. cerevisiae*. The lack of cysteine residues and a two-amino-acid insertion in the main domain differentiated *C. albicans* enolase from *S. cerevisiae* enolase. Immunofluorescence of whole *C. albicans* cells by using a mouse antiserum generated against the purified fusion protein showed that enolase is not located on the surface of *C. albicans*. Recombinant *C. albicans* enolase will be useful in understanding the pathogenesis and host immune response in disseminated candidiasis, since enolase is an immunodominant antigen which circulates during disseminated infections.

Enolase (2-phospho-D-glycerate hydrolyase; EC 4.2.11) is a central component of the glycolytic pathway, catalyzing the dehydration of 2-phosphoglycerate to create phosphoenolpyruvate. In addition, enolase catalyzes the reverse reaction during gluconeogenesis. Enolases from a variety of sources, including bacteria, yeasts, drosophila, amphibians, birds, plants, and humans, have been studied and found to be highly conserved (56). In addition to its well-known glycolytic function, enolase comprises an eye lens crystallin in avian species (60) and may function as a toxin in bacteria (2). The existence of tissue-specific forms of enolase in mammalian species and rodents suggests that enolase may have other specialized functions in these animals (26). In *Saccharomyces cerevisiae*, enolase and other glycolytic enzymes are the most abundant proteins in the cell (18, 29).

Enolase from *S. cerevisiae* is the most studied of all enolases with respect both to biochemical and biophysical characteristics and to genetic regulation. The apoenzyme has been crystallized, and the structure has been determined at 2.25-Å (0.225-nm) resolution (50). The regulation of enolase enzyme levels in *S. cerevisiae* is largely the result of transcriptional control. Enolase is encoded by two genes (*ENO1* and *ENO2*) whose transcription is mediated by multifunctional transcriptional modulators which bind to short sequences of DNA in the 5′ regions of these genes and to similar sequences in other genes (5, 8). In addition, gene-specific regulation occurs. The enzyme encoded by *ENO1* is increased during stationary phase (20), whereas enolase encoded by *ENO2* is increased when glucose is present in the growth media (33). Given the close evolutionary relationship between *S. cerevisiae* and the pathogenic yeast *Candida albicans*, it is likely that *C. albicans* enolase is an abundant protein whose expression is controlled by transcriptional modulators which control multiple genes. Unlike *S. cerevisiae*, *C. albicans* grows in both filamentous and yeast forms in its natural environment in animal hosts (34). Understanding the mechanism of growth transition is of interest because of the importance of the filamentous form in adhesion and invasion of host tissue. Interestingly, the conversion to the filamentous form is associated with growth phase and carbon source (34). Given that in vitro, growth stage and carbon source influence both morphogenesis in *C. albicans* and enolase gene expression in *S. cerevisiae*, it is possible that factors which control expression of enolase in *C. albicans* are also involved in regulating the yeast-to-hypha conversion.

C. albicans is an opportunistic fungal pathogen that is found colonizing mucosal surfaces of normal individuals (34). When host defenses are impaired, the natural invasive potential of the filamentous growth form leads to overgrowth and infection. Hematogenously disseminated infections frequently complicate medical management of leukemia, organ transplantation, and other conditions, leading to high mortality rates, prolonged hospitalization, and expense (59). A decrease in T-cell immunity, such as occurs with AIDS, is associated with oral, esophageal, and other mucosal infections (34).

Despite their potential importance in chemotherapy, growth control, pathogenesis, and host response, very little information is available on the glycolytic enzymes of *C. albicans*. None of their genes have been cloned. We have

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cloned and sequenced a cDNA for *C. albicans* enolase which includes the entire coding region, as judged from comparison with enolase genes from other organisms. Comparison of the predicted *C. albicans* enolase to the sequence of crystallized *S. cerevisiae* enolase showed extensive homology in regions of secondary structure, illustrating the similarity in architecture of the two enolases. mRNA hybridizing with the cloned DNA was found in the hyphal form as well as in the yeast form of *C. albicans*. The high degree of codon bias and the strong hybridization signal in Northern (RNA) blot analysis indicated that *C. albicans* enolase is a highly expressed gene. In addition, the protein product of the cloned cDNA has been purified as a recombinant protein fused to glutathione *S*-transferase (GST) and has been shown to have enolase enzymatic activity.

MATERIALS AND METHODS

Reagents. Restriction endonucleases, DNA polymerase, T4 DNA ligase, rabbit muscle lactate dehydrogenase, and rabbit muscle pyruvate kinase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Restriction enzymes were purchased from Bethesda Research Laboratories (BRL), Gaithersburg, Md. Sequenase and sequencing reagents were purchased from U.S. Biochemical, Cleveland, Ohio. [^{125}I]protein A, [^{35}S]methionine, [α-^{32}P]dATP, and [α-^{32}P]dGTP were purchased from Dupont, Wilmington, Del. Random hexamers for random primer labeling and the pGEX vector were from Pharmacia, Piscataway, N.J. Nitrocellulose was purchased from Schleicher & Schuell, Inc., Keene, N.H. Immobilon was purchased from Millipore, Corp., Bedford, Mass. Enolase from *S. cerevisiae*, NADH, ADP glutathione, and glutathione-conjugated agarose beads were purchased from Sigma Corp., St. Louis, Mo. The Qiagen reagents were purchased from Qiagen, Inc., Studio City, Calif., the BCA protein assay kit was purchased from Pierce Chemical Co., Rockford, Ill., Western immunoblotting reagents were purchased from Zymed Corp., San Francisco, Calif., and the Directigen$_{1\text{-}2\text{-}3}$ assay was purchased from Fisher Scientific, Pittsburgh, Pa. Protein molecular weight standards were from Promega Corp., Madison, Wis., and BRL. DNA and RNA standards were from BRL. Geneclean was from Bio 101, Inc., La Jolla, Calif. Tissue culture medium 199 was from GIBCO, Grand Island, N.Y. Ribi adjuvant was from Ribi Inc., Hamilton, Mont.

Fungal and bacterial strains. *C. albicans* 441B and SC5314 are clinical isolates which have been previously described (22, 53). *Escherichia coli* XL-1 Blue was purchased from Stratagene, and *E. coli* NM22 was purchased from International Biotechnologies, Inc., New Haven, Conn. *E. coli* DH5α was used as a host strain for pGEX and has been described previously (47).

Antiserum. Polyvalent antiserum to *C. albicans* was obtained from rabbits immunized with formalin-killed hyphal forms of *C. albicans* 441B (53). Anti-*E. coli* activities were removed by adsorption with an *E. coli*-λ ZAP crude extract as detailed in the Stratagene manual. Polyvalent antiserum to the recombinant fusion protein was produced by intraperitoneal injection of mice with 0.1 mg of recombinant fusion protein combined with the Ribi adjuvant system as an initial dose followed by two subsequent injections at 10 and 17 days. Animals were sacrificed 5 days following the final injection of fusion protein. Serum samples from five mice were pooled for immunoblotting. Serum samples from three unimmunized animals were pooled and used as a control.

Construction of a cDNA library. *C. albicans* SC5314 in early hyphal growth was used as a source of mRNA for library construction. Organisms were first grown to stationary phase in 1% yeast extract–2% Bacto Peptone–2% dextrose (YEPD) at 30°C for 48 h and then washed twice with 0.15 M NaCl containing 0.01 M phosphate, pH 7.4 (phosphate-buffered saline). Hyphal growth was then induced by placing stationary-phase yeast cells (6.8×10^6 organisms per ml) into medium 199 without serum, adjusted to pH 7.0. After incubating for 1.5 h at 37°C, over 95% of the cells had germ tubes of average length of one yeast cell diameter. Polyadenylated mRNA from the 1-h hyphal cultures was prepared by standard protocols (37, 52).

First- and second-strand cDNA synthesis carried out with this template mRNA (10 μg), cloning of the cDNA into the *Eco*RI sites of the expression vector λ ZAP (47), and production of primary and amplified libraries were performed by Stratagene, La Jolla, Calif. The *C. albicans* cDNA library contained 2.4×10^6 clones, 95% of which were recombinants, resulting in a final cloning efficiency of 2.4×10^5 clones per mg of mRNA. This is within the expected efficiency for yeast cDNA library production (14).

Screening of the cDNA library. Production of plaques containing recombinant *C. albicans* proteins and immunoscreening were done according to procedures in the Stratagene manual. Filters were treated with antiserum (1:100 dilution) to *C. albicans* and then with [^{125}I]protein A (0.05 μCi/ml). This initial screening of the cDNA library resulted in the identification of eight plaques which reacted with the antiserum. The plaques from the initial screening were purified by plating at low density and rescreening with antiserum until all plaques were positive.

Analysis of recombinant clones. Lambda DNA was prepared from liquid lysates as described in the Qiagen manual. The lambda DNA was then tested for the presence of cDNA inserts by restriction enzyme digests as instructed by the manufacturer. Molecular weights were determined by using *Hin*dIII-digested lambda DNA to generate a standard curve.

Production and purification of fusion protein. An *Xba*I-*Xho*I cDNA fragment containing the 1.4-kbp cDNA insert was first cloned into pIBI sequencing vectors (see below). A cDNA fragment for subcloning into pGEX-1 was created by digestion of p24eno with *Xho*I, creation of blunt ends with DNA polymerase, and digestion with *Bam*HI prior to cloning into the polylinker region of *Bam*HI- and *Sma*I-digested pGEX-1 (48). Linearized pGEX-1 and the 1.4-kbp cDNA insert were purified by agarose gel electrophoresis and processing with Geneclean.

Plasmid DNA from ampicillin-resistant transformants of *E. coli* DH5α was analyzed by restriction digestion to identify strains with plasmids containing inserts of the appropriate size. Induction and purification of fusion proteins were performed as described by others (47, 55). GST was prepared from control strains transformed with pGEX-1 alone. Proteins were electrophoresed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (23). Gels were stained with Coomassie blue, and the molecular weight of the fusion protein was determined from its relative mobility compared with the relative mobilities of purchased protein standards. The logarithms of the molecular weights of the purchased standards were plotted versus relative mobilities. Exponential least-squares curve fits ($r = 0.98$) were generated by using Kaleidograph (Synergy Software, Reading, Pa.). The standard error of the estimates was 1,070 molecular weight.

Immunoblotting. (i) With rabbit antiserum. Immunoblotting was done by standard techniques (17). Anti-*C. albicans*

antiserum or normal rabbit serum was used at a 1:150 dilution in blocking solution (50 mM Tris-HCl [pH 7.4]–200 mM NaCl–0.1% Tween 20 [TBST] containing 1% skim milk). Rabbit immunoglobulin G (IgG) bound to the blotted proteins was detected by using a kit purchased from Zymed that consisted of a biotinylated, affinity-purified goat anti-rabbit IgG and an alkaline phosphatase-conjugated streptavidin detection system. Proteins were visualized by staining duplicate membranes for 10 min with amido black (40).

(ii) With mouse antiserum. A *C. albicans* crude extract and *S. cerevisiae* enolase were electrophoresed along with the fusion protein and GST. A crude extract of *C. albicans* SC5314 was prepared by inoculating YPD (30 ml) with a fresh colony and growing the cells overnight at 30°C with shaking at 250 rpm. One milliliter of the overnight culture was used to inoculate 100 ml of YPD, and the culture was grown until early log phase was reached (60 Klett units). Cells were pelleted at 4,000 × *g* and washed two times in 0.01 M Tris-HCl (pH 7.0), and the final pellet was resuspended in 1.0 ml of 0.01 M Tris-HCl (pH 7.0) prior to freezing at −70°C overnight. Phenylmethylsulfonyl fluoride (8 μl of a 0.1 M solution in ethanol) and 2.5 g of acid-washed glass beads were added to the thawed pellet, which was then vortexed with glass beads (0.45-μm diameter) in cycles of 30-s pulses followed by 1 min on ice. Eight cycles were performed prior to addition of additional glass beads (2 g); then five more cycles of vortexing and ice incubation were performed. Microscopic analysis showed that the majority of cells were lysed. The broken cells were removed by centrifugation for 10 min at 4,000 × *g* in the cold; the supernatant was aspirated and stored at −20°C prior to electrophoresis. Protein concentration was estimated by using the BCA kit, with bovine serum albumin as a standard. Electrophoresis and blotting were performed as described above except that polyvinylidene difluoride membranes were blocked with 2% skim milk, the primary antibody was immune mouse serum or normal mouse serum (1:200 dilution), and the second antibody consisted of biotinylated rabbit anti-mouse IgG, IgA, and IgM (heavy chain plus light chain).

Enolase enzyme assay. Enolase enzymatic activity was determined in the coupled assay described by Maitra and Lobo (29), with slight modifications. The reaction mix consisted of 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM EDTA, 2 mM NADH, 1.0 mM ADP, 10 U of lactate dehydrogenase (EC 1.1.1.27), 10 U of pyruvate kinase (EC 2.7.1.40), and various amounts of *S. cerevisiae* enolase (EC 4.2.1.11), fusion protein, or GST. One unit of enolase activity was defined as the conversion of 1 mmol of NADH to NAD per min, based on an extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1} at 30°C. The reaction mixture without added substrate gave no measurable change in optical density.

Radioimmunoprecipitation. In vivo radiolabeling of *C. albicans* with [^{35}S]methionine and radioimmunoprecipitations were performed as previously described (54, 55), using Zymolyase digests of the hyphal growth form. To determine whether the fusion protein inhibited the reaction of the anti-*C. albicans* antiserum with a radiolabeled protein, the fusion protein (5 μg) was added to the antigen-antibody mixture. GST (5 μg) was used in control reactions. Molecular weights were determined as described above.

Immunofluorescence. Indirect immunofluorescence was performed on whole, formalin-killed hyphal cells as previously described (53). Mouse anti-fusion protein antiserum and normal mouse serum (negative control) were used at dilutions of 1:10 and 1:100. Affinity-purified fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (1:20 dilution) was used as the second antibody. As a positive control, organisms were stained with the polyvalent rabbit anti-*C. albicans* antiserum (1:100 dilution) with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG as the second antibody.

Northern blot analysis. Procedures for growing organisms in yeast or hyphal form in modified Lee's medium and extraction of RNA, electrophoresis, blotting, and hybridization conditions have been previously described (52). Nitrocellulose blots were probed with 10^6 cpm of the purified, radiolabeled *Xba*I-*Xho*I 1.4-kbp cloned cDNA or with the radiolabeled *C. albicans* actin gene (a generous gift of S. Riggsby) labeled by the random primer method (10, 11). The molecular weight of the RNA hybridizing to the 1.4-kbp cDNA probe was determined on the basis of a standard curve, using BRL RNA molecular weight standards. Exponential least-squares curve fits ($r = 0.99$) were generated by using Kaleidograph (Synergy Software). The intensities of the autoradiographic bands were measured with an LKB Ultrascan XL enhanced laser densitometer and GelScan XL 2.1.

Sequencing. The purified 1.4-kbp *Xba*I-*Xho*I cDNA and 260-bp *Xba*I-*Sal*I cDNA fragments (see Fig. 2) were subcloned in both orientations into the sequencing vectors pIBI24 and pIBI25 to produce plasmids p24eno and p25eno. The complete sequences of both strands were determined by using the dideoxy-chain termination method on purified single-stranded DNA. When required, oligonucleotide probes were prepared in an automated Milligen Cyclone Plus synthesizer and used to continue sequencing. Sequence analysis was carried out by using the DNA Inspector IIE program (Textco, Boston, Mass.) and Software version 7.0 from the Genetics Computer Group at the University of Wisconsin (9). Sequences of enolase proteins from various organisms were found in the SwissProt data base, release 17.0.

Disseminated candidiasis assay. The Directigen$_{1\text{-}2\text{-}3}$ test for disseminated candidiasis was carried out according to the directions of the manufacturer. The fusion protein and GST were diluted (0.2 to 20 nM) in buffer containing 0.01 M Tris-HCl (pH 8.0), 5 mM EDTA, and 1% skim milk prior to assay.

Nucleotide sequence accession number. The GenBank accession number for the cDNA sequence reported here is M93712.

RESULTS

Immunoselection of recombinant cDNAs of *C. albicans*. Eight plaques that produced immune reactions among the 8 × 10^4 screened were isolated. Restriction enzyme digestion analysis of DNA from the positive plaques suggested that they all contained identical 1.4-kbp inserts (not shown). One clone, λC1.d, was chosen for further analysis. A restriction map of the recombinant clone λC1.d is shown in Fig. 1.

Immunoblot analysis of the recombinant fusion protein. The 1.4-kbp cDNA *Xba*I-*Xho*I insert was subcloned into the pGEX expression vector (48) to produce a polypeptide consisting of the protein product of the cDNA insert fused to the carboxy-terminal end of GST. *E. coli* sonic extracts were prepared from the transformants, and fusion proteins were purified on glutathione-containing agarose beads.

A fusion protein of molecular weight 68,400 was purified from *E. coli* strains which had been transformed with the recombinant pGEX plasmid. Western blot analysis showed

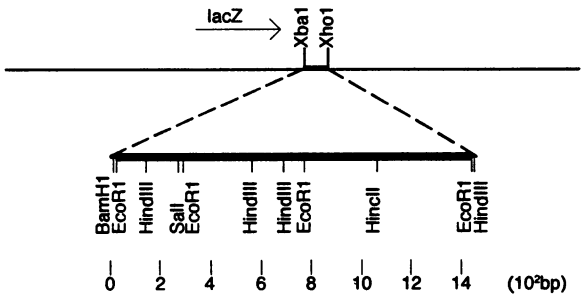

FIG. 1. Endonuclease restriction map of λC1.d. The positions of restriction sites and orientation of the insert relative to *lacZ* are shown. ——, λZAP DNA; ▬, *C. albicans* cDNA.

that as little as 0.01 μg of fusion protein reacted strongly with the anti-*C. albicans* antiserum used to screen the cDNA library, whereas 10 times as much GST was barely stained (Fig. 2B, lanes 5 to 7). Neither protein reacted with normal rabbit control serum (not shown).

Northern analysis. Northern transfers of total RNA from *C. albicans* growing in either yeast or hyphal form showed one distinct band when probed with the purified 1.4-kbp cDNA (Fig. 3A). The molecular size of this species was 1.5 kb. Given that the 1.4-kbp cDNA included approximately 100 bp outside the enolase coding region, the size of the mRNA from Northern blot analysis suggested that enolase mRNA contains approximately 200 nucleotides of nontranslated sequences.

To control for equivalent loading and transfer of RNA in Northern blotting, a duplicate blot was probed with the *C. albicans* actin gene (Fig. 3B). Yeast and hyphal growth phases contained roughly equivalent levels of *C. albicans* actin mRNA (Fig. 3B). The levels of mRNA hybridizing to the 1.4-kbp cDNA probe were at least fivefold higher than levels of actin mRNA in all four cultures, indicating that the cloned cDNA represents a highly expressed *C. albicans* mRNA. The higher levels of 1.5-kb mRNA than of actin mRNA were not a consequence of using duplicate blots, since a similar ratio was found when a single blot was probed first with the actin gene, stripped, and reprobed with the

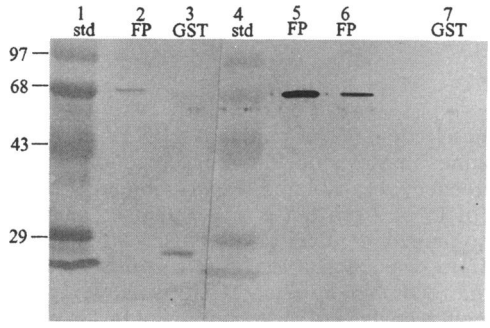

FIG. 2. Western blot analysis of the purified fusion protein. GST and the fusion protein (FP) were purified from *E. coli* sonic extracts on glutathione-agarose beads, electrophoresed on an SDS–10% polyacrylamide gel, and transferred electrophoretically to an Immobilon membrane prior to staining with the polyvalent antiserum to *C. albicans* (lanes 4 to 7) or with amido black to stain proteins (lanes 1 to 3). The proteins were loaded on the gel as follows: prestained molecular weight markers from BRL (lanes 1 and 4), 1 μg of GST (lanes 3 and 7), 0.1 μg of fusion protein (lanes 2 and 5), and 0.01 μg of fusion protein (lane 6). Sizes are indicated in kilodaltons.

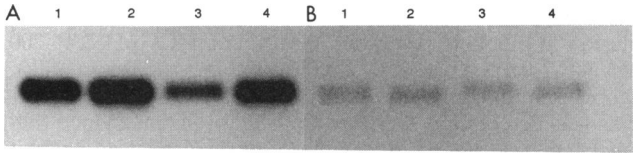

FIG. 3. Northern blot analysis of *C. albicans* RNA. Total RNA (20 μg per lane) prepared from *C. albicans* yeast (grown at 37°C, pH 4.5 [lane 1], 30°C, pH 4.5 (lane 2), or 30°C, pH 6.5 [lane 4]) or hyphal (grown at 37°C, pH 6.5 [lane 3]) forms was electrophoresed in a formaldehyde-containing 1.2% agarose gel before being transferred to nitrocellulose. Duplicate lanes of each RNA sample were run on the same gel. One blot (A) was probed with the 1.4-kbp cDNA, while the duplicate blot (B) was probed with the *C. albicans* actin gene. Molecular weights were determined by using RNA standards of 9.49, 7.46, 4.4, 2.37, 1.35, and 0.24 kb to generate a standard curve.

1.4-kb cDNA (not shown). Both yeast and hyphal growth phases contained mRNA which hybridized to the 1.4-kb cDNA, although the relative levels of enolase mRNA in yeast and hyphae varied depending on the media used and the growth stage of the yeasts used to initiate hypha formation (not shown).

DNA sequence. The 1.4-kbp *Xba*I-*Xho*I and the 266-bp *Xba*I-*Sal*I fragments (Fig. 1) were subcloned into the single-stranded sequencing vectors pIBI24 and pIBI25 to allow sequencing of both strands. One long open reading frame of 1,320 bp, beginning with a methionine codon and ending with a stop codon, was found (Fig. 4). Alignment of the sequence with the *S. cerevisiae* enolase sequences strongly suggested that the indicated methionine is the initial amino acid in the protein (Fig. 5). The reading frame predicted a protein of molecular weight 47,178 with an isoelectric point 5.6 consisting of 440 amino acids. The sequence also contained 10 nucleotides 5′ of the initial methionine and 106 nucleotides of 3′ flanking sequences (not shown). The sum of the base pairs for coding plus flanking sequences is in agreement with the size of the purified cDNA (1.4 kbp).

Computer searches of data bases of known proteins revealed that the amino acid sequence had a high degree of identity (76 to 78% identical amino acids) and 87% similarity (when matching conserved amino acids) to the predicted enolase proteins of the *ENO1* and *ENO2* genes of *S. cerevisiae* (Table 1). Comparison of the predicted *C. albicans* enolase protein with enolases from other organisms (16) showed lesser degrees of similarity (Table 1). The *C. albicans* predicted protein sequence includes 148 of the 151 amino acids reported to be conserved in enolases from several organisms (56). However, one (Thr-332) of the three amino acids (Val-178, Asp-219, and Thr-332) in *C. albicans* enolase that differed from the other enolases is also threonine at the corresponding residue in the recently published sequence of maize enolase (24).

To optimize alignment of the *C. albicans* sequence with the *S. cerevisiae* ENO1 sequence, it was necessary to introduce one gap after Ala-314 of the *S. cerevisiae* ENO1 sequence (Fig. 5). If the N-terminal methionine is cleaved as in *S. cerevisiae* enolase (7), *C. albicans* enolase would be predicted to have one additional amino acid at its N terminus relative to *S. cerevisiae* enolase. An interesting feature of the predicted *C. albicans* enolase was the absence of cysteine residues, in comparison with the single cysteine of *S. cerevisiae* enolase following the active site aspartate residue at position 248. Like *S. cerevisiae* enolase, *C. albicans* enolase was seen to have a two-amino-acid insertion (Lys at posi-

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                                                30                                                  60
ATG TCT TAC GCC ACT AAA ATC CAC GCC AGA TAC GTC TAC GAC TCC AGA GGT AAC CCA ACC GTT GAA GTT GAT TTC
Met Ser Tyr Ala Thr Lys Ile His Ala Arg Tyr Val Tyr Asp Ser Arg Gly Asn Pro Thr Val Glu Val Asp Phe
                                    10                                                  20

                    90                                                 120                                                 150
ACC ACC GAC AAA GGT TTA TTC AGA TCA ATT GTC CCA TCT GGT GCC TCT ACT GGT GTC CAC GAA GCT TTG GAA TTG
Thr Thr Asp Lys Gly Leu Phe Arg Ser Ile Val Pro Ser Gly Ala Ser Thr Gly Val His Glu Ala Leu Glu Leu
                    30                                                 40                                                  50

                                                180                                                 210
AGA GAT GGT GAC AAA TCC AAA TGG TTA GGT AAA GGT GTT TTG AAA GCC GTT GCC AAT GTT AAT GAC ATC ATT GCC
Arg Asp Gly Asp Lys Ser Lys Trp Leu Gly Lys Gly Val Leu Lys Ala Val Ala Asn Val Asn Asp Ile Ile Ala
                                    60                                                  70

                    240                                                270                                                 300
CCA GCT TTA ATA AAA GCC AAG ATC GAT GTT GTC GAC CAA GCT AAG ATT GAT GAA TTC TTG TTG TCC TTG GAC GGT
Pro Ala Leu Ile Lys Ala Lys Ile Asp Val Val Asp Gln Ala Lys Ile Asp Glu Phe Leu Leu Ser Leu Asp Gly
                    80                                                 90                                                  100

                                                330                                                 360
ACT CCA AAC AAA TCC AAA TTG GGT GCC AAT GCT ATC TTG GGT GTT TCT TTG GCT GCT GCC AAT GCT GCC GCT GCT
Thr Pro Asn Lys Ser Lys Leu Gly Ala Asn Ala Ile Leu Gly Val Ser Leu Ala Ala Ala Asn Ala Ala Ala Ala
                                    110                                                 120

                    390                                                420                                                 450
GCT CAA GGC ATT CCA TTG TAC AAA CAC ATT GCC AAC ATT TCC AAT GCC AAG AAA GGT AAA TTC GTT TTG CCA GTT
Ala Gln Gly Ile Pro Leu Tyr Lys His Ile Ala Asn Ile Ser Asn Ala Lys Lys Gly Lys Phe Val Leu Pro Val
                    130                                                140                                                 150

                                                480                                                 510
CCA TTC CAA AAC GTT TTG AAC GGT GGT TCC CAT GCT GGT GGT GCT TTA GCT TTC CAA GAA TTT ATG ATT GCC CCA
Pro Phe Gln Asn Val Leu Asn Gly Gly Ser His Ala Gly Gly Ala Leu Ala Phe Gln Glu Phe Met Ile Ala Pro
                                    160                                                 170

                    540                                                570                                                 600
ACT GGT GTC TCC ACT TTC TCT GAA GCT TTG AGA ATT GGT TCA GAA GTT TAC CAC AAC TTG AAA TCT TTG ACC AAG
Thr Gly Val Ser Thr Phe Ser Glu Ala Leu Arg Ile Gly Ser Glu Val Tyr His Asn Leu Lys Ser Leu Thr Lys
                    180                                                190                                                 200

                                                630                                                 660
AAG AAA TAC GGT CAA TCC GCT GGT AAC GTC GGT GAC GAA GGT GGT GTT GCT CCA GAT ATC AAA ACT CCA AAG GAA
Lys Lys Tyr Gly Gln Ser Ala Gly Asn Val Gly Asp Glu Gly Gly Val Ala Pro Asp Ile Lys Thr Pro Lys Glu
                                    210                                                 220

                    690                                                720                                                 750
GCT TTG GAC TTG ATC ATG GAT GCC ATT GAC AAA GCC GGT TAC AAA GGT AAG GTT GGT ATT GCC ATG GAT GTT GCT
Ala Leu Asp Leu Ile Met Asp Ala Ile Asp Lys Ala Gly Tyr Lys Gly Lys Val Gly Ile Ala Met Asp Val Ala
                    230                                                240                                                 250

                                                780                                                 810
TCA TCT GAA TTC TAC AAG GAC GGT AAA TAC GAC TTG GAC TTT AAA AAC CCA GAA TCC GAC CCA TCT AAA TGG TTG
Ser Ser Glu Phe Tyr Lys Asp Gly Lys Tyr Asp Leu Asp Phe Lys Asn Pro Glu Ser Asp Pro Ser Lys Trp Leu
                                    260                                                 270

                    840                                                870                                                 900
TCT GGC CCA CAA TTG GCT GAC TTA TAT GAA CAA TTG ATT TCC GAA TAC CCA ATT GTT TCT ATT GAA GAT CCA TTC
Ser Gly Pro Gln Leu Ala Asp Leu Tyr Glu Gln Leu Ile Ser Glu Tyr Pro Ile Val Ser Ile Glu Asp Pro Phe
                    280                                                290                                                 300

                                                930                                                 960
GCT GAA GAT GAC TGG GAT GCT TGG GTC CAC TTC TTT GAA AGA GTT GGT GAC AAG ATC CAA ATT GTC GGT GAT GAT
Ala Glu Asp Asp Trp Asp Ala Trp Val His Phe Phe Glu Arg Val Gly Asp Lys Ile Gln Ile Val Gly Asp Asp
                                    310                                                 320

                    990                                               1020                                                1050
TTG ACT GTC ACT AAC CCT ACC AGA ATC AAG ACT GCC ATT GAA AAG AAA GCC GCT AAT GCT TTG TTG TTG AAG GTT
Leu Thr Val Thr Asn Pro Thr Arg Ile Lys Thr Ala Ile Glu Lys Lys Ala Ala Asn Ala Leu Leu Leu Lys Val
                    330                                                340                                                 350

                                               1080                                                1110
AAC CAA ATT GGT ACT TTG ACT GAA TCT ATA CAA GCT GCT AAC GAT TCT TAC GCT GCT GGT TGG GGT GTC ATG GTT
Asn Gln Ile Gly Thr Leu Thr Glu Ser Ile Gln Ala Ala Asn Asp Ser Tyr Ala Ala Gly Trp Gly Val Met Val
                                    360                                                 370

                   1140                                               1170                                                1200
TCC CAC AGA TCC GGT GAA ACC GAA GAT ACT TTC ATT GCT GAC TTG TCA GTT GGT TTA AGA TCT GGT CAA ATC AAG
Ser His Arg Ser Gly Glu Thr Glu Asp Thr Phe Ile Ala Asp Leu Ser Val Gly Leu Arg Ser Gly Gln Ile Lys
                    380                                                390                                                 400

                                               1230                                                1260
ACT GGT GCT CCA GCT AGA TCT GAA AGA TTG GCC AAA TTG AAC CAA ATC TTG AGA ATC GAA GAA GAA TTA GGT TCT
Thr Gly Ala Pro Ala Arg Ser Glu Arg Leu Ala Lys Leu Asn Gln Ile Leu Arg Ile Glu Glu Glu Leu Gly Ser
                                    410                                                 420

                   1290                                               1320
GAA GCT ATC TAC GCT GGT AAA GAT TTC CAA AAG GCT TCT CAA TTG TAA
Glu Ala Ile Tyr Ala Gly Lys Asp Phe Gln Lys Ala Ser Gln Leu Ter
                    430                                                440
```

FIG. 4. DNA sequence and predicted amino acid sequence of the *C. albicans* enolase cDNA.

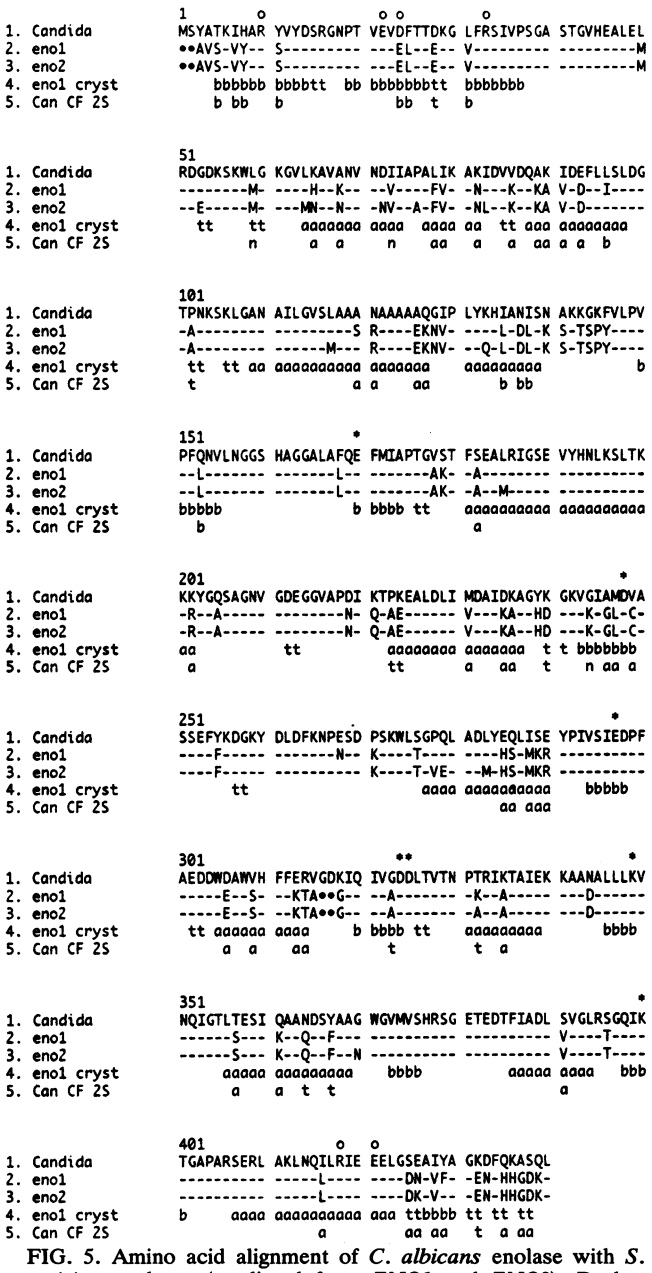

FIG. 5. Amino acid alignment of *C. albicans* enolase with *S. cerevisiae* enolases (predicted from *ENO1* and *ENO2*). Dashes indicate residues identical to those of the *C. albicans* predicted enolase. Line 4 shows the location of the α helices and β sheets seen from the crystal structure of *S. cerevisiae* enolase A (25), the protein product of *ENO1*. Line 5 is a Chou-Fasman analysis of secondary structure of *C. albicans* enolase residues which differ from the corresponding *S. cerevisiae* residues and are in regions of crystal-determined secondary structure. Asterisks indicate residues which are important for catalysis, and open circles indicate residues involved in subunit interactions. Abbreviations: a, α helix; b, β strand; t, turn; n, no secondary structure by Chou-Fasman analysis.

tions 142 and 143) relative to vertebrate enolases, although the corresponding residues in *S. cerevisiae* enolase were Lys and Thr.

A partial *C. albicans* cDNA clone with similarity to *S. cerevisiae* has been reported by Franklyn et al. (12). Their sequence corresponds to amino acids 95 to 251 of our sequence, or about one-third of the protein, and differs at positions 209, 210, and 251 in our sequence. At those positions, our sequence is identical to that reported for both *S. cerevisiae* enolase proteins (Fig. 5).

TABLE 1. Percent amino acid similarity and identity between *C. albicans* enolase and enolases from other organisms[a]

Organism	Reference	Gene	% Similarity to *C. albicans* enolase	% Identity to *C. albicans* enolase
S. cerevisiae	19	*ENO1*	87.59	78.16
		ENO2	86.90	76.32
Frog	46	*ENO*	78.27	64.50
Drosophila	1	*ENO*	79.02	67.13
Duck	60	*ENOα*	79.20	66.35
Human	13	*ENOα*	78.45	65.10
	6, 39	*ENOβ*	77.62	65.03
	32, 36, 56	*ENOγ*	78.37	65.58
Rat	43	*ENOα*	77.51	65.34
	35	*ENOβ*	76.45	63.63
	42, 44	*ENOγ*	78.14	65.58
Mouse	21	*ENOα*	76.81	64.87
	21	*ENOγ*	78.19	65.66
Chicken	41	*ENOβ*	76.22	62.47
E. coli[b]	58	*ENO*	74.59	63.11

[a] Percent similarities and identities were calculated by Genetics Computer Group software on the basis of algorithms written by Gribskov and Burgess (16).

[b] Data based on a fragment of the enolase gene encoding 123 amino acids.

The codon usage of the *C. albicans* genes was highly biased and was almost identical to the codon bias found for the two EF-1α genes of *C. albicans* (Table 2) (52). There were no differences in codon usage between enolase and EF-1α genes in the most frequently used codons. However, minor differences were that the enolase gene utilized codons for amino acids Gly, Ile, Pro, Ser, and Tyr which were not used in the EF-1α genes. In contrast, one codon for Thr, ACA, was used by the EF-1α genes but not by enolase genes. The high degree of codon bias for *C. albicans* enolase is consistent with its being a highly expressed protein, utilizing the isoacceptor tRNAs which are in greatest abundance in *C. albicans*.

Assay of the fusion protein for enolase enzymatic activity. The high degree of homology of the cloned cDNA with enolase genes from other organisms suggested that the purified 68,400-molecular-weight fusion protein might have enolase enzyme activity. The fusion protein as well as purchased enolase from *S. cerevisiae*, but not GST, had enolase enzyme activity (Table 3). In addition, the rate of the reaction doubled with doubling of the fusion protein concentration. The specific enolase activities for the fusion protein and GST were 49.7 ± 3.7 and 0.7 U/mg, respectively.

Radioimmunoprecipitation. To determine whether the fusion protein cross-reacted with a *C. albicans* protein, the ability of the fusion protein to compete with components of a radiolabeled Zymolyase digest of hyphal cells for binding to anti-*C. albicans* antibodies was tested. The precipitation of a single *C. albicans* protein was completely inhibited by the fusion protein (Fig. 6A, lane 2) but not by GST (lane 1). The molecular weight of the inhibited protein was estimated to be 47,000 on the basis of its mobility relative to those of molecular weight standards. The apparent comigration of this protein with the 42,700-molecular-weight markers reflects the anomalous slow migration of the 42,700- and

TABLE 2. Comparison of frequencies of codon usage of EF-1α and enolase genes from *C. albicans*

Amino acid	Codon	No. of times used in:			Amino acid	Codon	No. of times used in:			Amino acid	Codon	No. of times used in:		
		TEF1	*TEF2*	*ENO*			*TEF1*	*TEF2*	*ENO*			*TEF1*	*TEF2*	*ENO*
Ala	GCT	28	28	33	Gly	GGT	43	43	37	Pro	CCT	0	0	1
	GCC	5	5	19		GGC	0	0	2		CCC	0	0	0
	GCA	0	0	0		GGA	0	0	0		CCA	24	24	16
	GCG	0	0	0		GGG	0	0	0		CCG	0	0	0
Arg	CGT	0	0	0	His	CAT	2	2	1	Ser	TCT	10	9	16
	CGC	0	0	0		CAC	9	9	6		TCC	9	10	12
	CGA	0	0	0							TCA	0	0	4
	CGG	0	0	0	Ile	ATT	20	19	17		TCG	0	0	0
	AGA	16	16	12		ATC	12	13	12		AGT	0	0	0
	AGG	0	0	0		ATA	0	0	2		AGC	0	0	0
Asn	AAT	5	5	6	Leu	TTA	3	3	7	Thr	ACT	17	17	13
	AAC	12	12	12		TTG	18	18	31		ACC	16	16	6
						CTT	0	0	0		ACA	1	1	0
Asp	GAT	12	12	15		CTC	0	0	0		ACG	0	0	0
	GAC	13	13	17		CTA	0	0	0					
						CTG	0	0	0	Trp	TGG	6	6	5
Cys	TGT	8	8	0										
	TGC	0	0	0	Lys	AAA	30	30	23	Tyr	TAT	0	0	1
						AAG	22	22	14		TAC	9	9	12
Gln	CAA	9	9	14										
	CAG	0	0	0	Met	ATG	9	9	5	Val	GTT	29	29	19
											GTC	16	16	10
Glu	GAA	30	30	25	Phe	TTT	1	1	3		GTA	0	0	0
	GAG	0	0	0		TTC	14	14	12		GTG	0	0	0

40,000-molecular-weight markers relative to migration of the other proteins in the standard curve (Fig. 6B). Similar results were seen with use of extracts from yeast forms and with two different strains of *C. albicans* (not shown).

Immunofluorescence. To determine whether *C. albicans* enolase was present on the surface, immunofluorescence of whole, formalin-killed hyphal cells was performed, using antiserum prepared in mice immunized with the fusion protein. As expected, the mouse antiserum contained antibodies to the fusion protein, schistosomal GST, and purchased *S. cerevisiae* enolase (Fig. 7B). Importantly, the mouse antiserum identified a protein, most likely *C. albicans* enolase, from a crude extract of *C. albicans* migrating slightly faster than *S. cerevisiae* enolase (Fig. 7B). The antiserum apparently did not detect *C. albicans* GST, since no proteins in the appropriate size range were recognized by the mouse antiserum. Most of the proteins in the crude extract of *C. albicans* did not react with the mouse antiserum; however, weak blotting was observed with two proteins of higher molecular weight. The protein which migrated close to the 68-kDa molecular size marker was found to be related to GST by its reaction with antiserum to GST (not shown). The other protein probably represents an aggregate of enolase or a protein cross-reactive with enolase. Normal mouse serum reacted weakly with the fusion protein, possibly because of previous exposure to fungal enolases (Fig. 7C). The mouse antiserum to the fusion protein did not stain whole organisms in the immunofluorescence assay, indicating that *C. albicans* enolase is not surface located. Organisms stained with the positive control rabbit anti-*C. albicans* polyvalent antiserum fluoresced brightly.

TABLE 3. Measurement of enolase enzyme activity[a] of the *C. albicans* GST fusion protein

Protein	Amt (μg)	μg of NADH converted to NAD/min
C. alibcans		
GST fusion protein	0.072	4.44
	0.145	8.69
	0.29	16.0
	0.58	31.4
GST	0.60	0.40
S. cerevisiae enolase	0.25	28.55
	0.5	55.75
	1.0	103

[a] Enolase activity was measured via a coupled assay in the presence of excess pyruvate kinase and lactate dehydrogenase. The conversion of NADH to NAD was measured by monitoring the decrease in A_{340} upon addition of 2-phosphoglycerate to the reaction mix.

Reactivity of the fusion protein in a commercial assay for disseminated candidiasis. A recently developed test specific for disseminated candidiasis is based on a monoclonal antibody to a 48,000-molecular-weight *C. albicans* antigen cross-reactive with enolase from *S. cerevisiae* (30). To determine whether the cloned *C. albicans* 1.4-kbp cDNA encoded the same antigen, the recombinant fusion protein was tested in this assay. The purified fusion protein was positive at a concentration of only 0.8 nM, which compared favorably with the reported sensitivity of 2 nM (57). In contrast, GST at a concentration of 20 nM was negative. These results indicated that the recombinant enolase produced in vitro in this work was immunologically related to the antigen circulating in the serum of patients with disseminated candidiasis.

DISCUSSION

The identity of the cloned gene that we isolated as the *C. albicans* enolase gene was established in several ways. The DNA sequence was shown to have a high degree of similarity to enolase genes from other organisms. The predicted protein of 440 amino acids is within the size range reported for other enolases, i.e., 433 amino acids for the mammalian enzymes (56), 436 amino acids for the *S. cerevisiae* enzymes

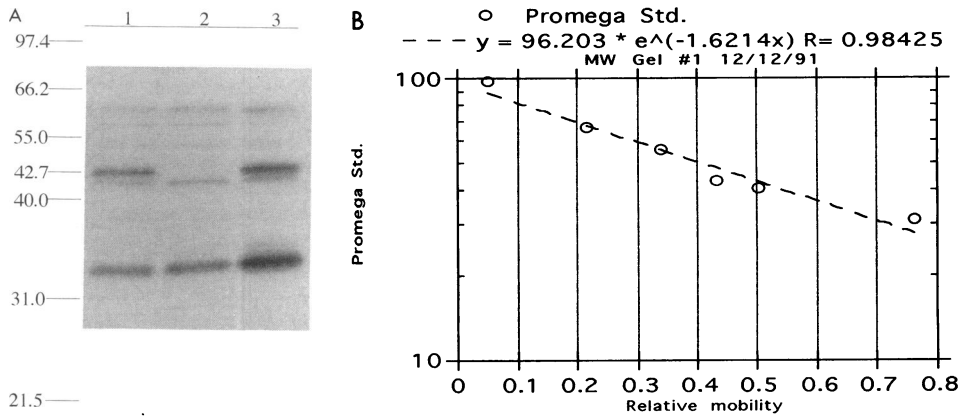

FIG. 6. Inhibition by the fusion protein of immunoprecipitation of a *C. albicans* antigen. (A) Radiolabeled Zymolyase digests of *C. albicans* (3×10^6 cpm) were mixed with anti-*C. albicans* antiserum (2 μl) and either fusion protein (lane 2), GST (lane 3), or no added proteins (lane 1). Immune complexes were purified by using formalin-killed staphylococci, eluted by boiling in 1× SDS sample buffer, and electrophoresed on an SDS–10% polyacrylamide gel. The fusion protein inhibited one radiolabeled *C. albicans* protein with an estimated molecular weight of 47,100 ± 1,070. Molecular weights are indicated in thousands. (B) Standard curve used to generate molecular weights. Std., standard; mw, molecular weight.

(33), and 444 and 446 amino acids for tomato and maize enolases, respectively (24, 56). As for most other genes of *C. albicans*, including the EF-1α, tubulin, and actin genes (27, 49, 52), the *C. albicans* enolase gene has more similarity to the corresponding genes of *S. cerevisiae* than to those of other organisms used in comparison. An exception to this generalization is the *C. albicans* calmodulin gene (45).

Additional data establishing the identity of the cDNA clone as encoding *C. albicans* enolase was provided by the demonstration of enolase enzymatic activity of a recombinant fusion protein produced from a clone of the 1.4-kbp cDNA fused to the C terminus of GST. Conversion of substrate to product was linear with time (not shown) and fusion protein concentration. It can be estimated that the GST portion of the fusion protein inhibited *C. albicans* enolase activity only by about 28%, given the knowledge that the fusion protein is only 62% enolase and assuming, on the basis of sequence conservation between the two predicted proteins, that enolases of *C. albicans* and *S. cerevisiae* have comparable specific activities.

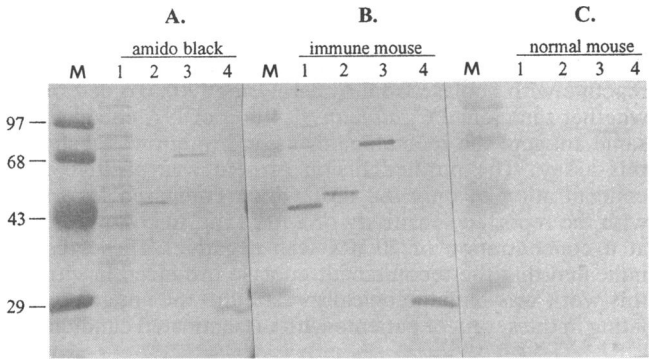

FIG. 7. Immunoblot analysis of *C. albicans* and *S. cerevisiae* enolases, using antiserum to the fusion protein. *C. albicans* crude extract (4 μg; lanes 1), *S. cerevisiae* enolase (0.05 μg; lanes 2), fusion protein (0.05 μg; lanes 3), or GST (0.05 μg; lanes 4) was electrophoresed on an SDS–10% polyacrylamide gel, transferred to polyvinylidene difluoride membranes, and stained with amido black (A), mouse antiserum to the fusion protein (1:200 dilution) (B), or normal mouse serum (C). Lanes M contained BRL prestained molecular weight standards of 97.4, 68, 43, and 29 kDa (indicated on the left).

Evidence that the clone that we isolated encoded *C. albicans* enolase and not an enolase from some other organism was provided by the strong hybridization signal under conditions of high stringency in Northern blot analysis between *C. albicans* RNA and a radiolabeled probe from the cloned cDNA. In addition, there were no differences in codon usage between enolase cDNA and EF-1α genes in the most frequently used codons (52) (Table 2), suggesting that both enolase cDNA and EF-1α genes are from the same organism, *C. albicans*. Minor differences seen were the use in enolase cDNA of codons for amino acids Gly, Ile, Pro, Ser, and Tyr which are not used in the EF-1α genes. Also, one codon for Thr, ACA, was used by the EF-1α genes but not by the enolase gene. The high degree of codon bias for *C. albicans* enolase is consistent with its being a highly expressed protein, utilizing the isoacceptor tRNAs which are in greatest abundance in *C. albicans*.

C. albicans enolase cDNA showed a remarkable similarity in codon bias to enolase genes of *S. cerevisiae*, as is the case for EF-1α genes from the two fungi. However, one difference between the two fungi was found with lysine codons. Whereas *S. cerevisiae* is heavily biased to encode lysine predominantly with AAG (19), *C. albicans* uses both AAA and AAG, with a slight preference for AAA.

An analysis of the predicted *C. albicans* enolase showed strong conservation in regions of α helices, β sheets, and β turns, as determined by the crystal structure of apo-enolase A of *S. cerevisiae* (25, 26, 50) (Fig. 5). Excluding the 16 divergent amino acids at the C terminus, we found 80% identical amino acids between the two sequences in regions of β structure and 74% identity in regions of α helices. A Chou-Fasman analysis of the secondary structure of the predicted *C. albicans* enolase showed that most of the amino acids which differed from *S. cerevisiae* enolase in regions of α helices and β sheets were compatible with the secondary structure seen in the crystal (Fig. 5, line 5). On the basis of this analysis, 98% of the *C. albicans* amino acids in regions of crystal-determined β sheet were compatible with β sheets

and 87% of the *C. albicans* amino acids in regions of crystal-determined α helices were compatible with α helices.

The architecture of *C. albicans* enolase is directly comparable with that of *S. cerevisiae* enolase, as judged from the high similarity in regions of secondary structure of the two proteins. X-ray crystallographic analysis of *S. cerevisiae* apo-enolase A reveals a small N-terminal domain of α-plus-β structure, beginning with a three-stranded antiparallel meander and four helices, followed by a main catalytic domain consisting of an eightfold β-plus-α barrel similar to that of the triose phosphate isomerase model (TIM barrel) (3) but with ββαα $(\beta + \alpha)_6$ rather than the usual $(\beta + \alpha)_8$ topology (26, 50). Multiple hydrophobic interactions occur between the two domains, contributing to the compact globular structure for the molecule (50). The high conservation in *C. albicans* enolase in regions of β sheet (98%) reflects the functional role of these residues. The strands of β structure in the C-terminal main domain are situated in the center of the domain, with catalysis occurring at the carboxy end of the β barrel, whereas β strands in the N-terminal domain provide a framework for subunit interaction of the enolase dimer. The lesser conservation in regions of α helix (87%) reflects their noncatalytic, structural role surrounding the inner β strands of the main domain. The internal helices (helices G and H) are highly conserved in *C. albicans* enolase (residues 386 to 394 and 407 to 423). In addition to the conservation in secondary structure, amino acids predicted to participate in catalysis were present in the predicted *C. albicans* enolase. Also, loop regions (residues 38 to 49, 206 to 218, and 251 to 274) constituting minidomains which are postulated to guide the substrate to the active site, as well as those participating in ionic bonds, and a classic bulge (Asn-154 to Asn-157) (26, 50) were conserved in *C. albicans* enolase.

Some minor differences between *S. cerevisiae* enolase and the predicted *C. albicans* amino acid sequence which might cause subtle differences in overall structure were observed. At positions 134 to 138, Chou-Fasman analysis predicted β structure rather than α helix in *C. albicans* enolase. A Chou-Fasman analysis of the corresponding *S. cerevisiae* sequence in this location correctly predicted α helix, consistent with the crystal structure (not shown). Residues 134 to 138 are in the region joining the N-terminal domain to the C-terminal domain, where secondary structure differences could lead to different packing of the N- and C-terminal domains in the two fungal enolases. Three other minor differences between *C. albicans* and *S. cerevisiae* enolases were seen. First, although the active site residue Asp-248 was conserved in *C. albicans* enolase, the surrounding amino acids (243 to 249) were predicted to be in α-helical rather than β-strand conformation as found in the crystal structure. Given the conserved structural features related to catalysis in enzymes with β-plus-α barrel structures (3), it seems most likely that Chou-Fasman analysis incorrectly predicted the secondary structure in this location. Second, the amino acid insertions (Gly-316 and Asp-317) relative to the *S. cerevisiae* sequence occurred between the C-terminal end of α-helix D and the N-terminal (noncatalytic) end of the inner barrel and thus would not be predicted to have a sizable functional or structural effect. Third, the absence of a cysteine residue corresponding to the single cysteine residue in *S. cerevisiae* enolase adjacent to active-site residue Asp-248 probably does not adversely affect function, since vertebrate and plant enolases, like *C. albicans* enolase, have a valine (residue 249) at this position (56).

The apparent molecular weight (68,400) of the fusion protein is small compared with the molecular weight of 47,178 calculated from the DNA sequence and the molecular weight of GST (48). The reasons for this are unclear but could include anomalous migration on SDS-polyacrylamide gel electrophoresis or proteolytic processing of the fusion protein by *E. coli*. Restriction endonuclease analysis of the recombinant pGEX plasmid showed that the low molecular weight of the fusion protein was not caused by deletion of a portion of the DNA during cloning (not shown). The ability to purify the fusion protein on glutathione-conjugated agarose beads indicated that GST is present on the fusion protein. The fact that the fusion protein specifically inhibited radioimmunoprecipitation of a *C. albicans* protein of molecular weight 47,000 in immunoprecipitations using radiolabeled cell wall digests and the anti-*C. albicans* antiserum, along with an immunoblot showing that antiserum to the fusion protein bound to a protein of similar size in a crude extract of *C. albicans*, confirmed that the molecular weight of *C. albicans* enolase predicted by the cDNA sequence is correct.

Most organisms which have been studied have multiple genes for enolase (Table 1). Mammalians have three genes encoding isozymes of enolase with different tissue specificities which may interact with different macromolecules (25). *S. cerevisiae* has two differentially regulated, nontandem genes for enolase which encode polypeptides differing in 20 of 436 amino acids and which migrate as a doublet on SDS-polyacrylamide gels (19, 33). The reason only one polypeptide is seen in the immunoblot of *S. cerevisiae* enolase in Fig. 7B is that enolase B is lost during purification (4). The number of genes for enolase in *C. albicans* is unknown; however, our immunoblot and immunoprecipitation results suggest that *C. albicans* has only one enolase polypeptide. These results agree with those of Mason et al. showing that antibody to *S. cerevisiae* enolase precipitates a single polypeptide from in vitro translation products of *C. albicans* (30). The plants maize and *Arabidopsis* sp. have only one gene for enolase; however, tomato has multiple enolase genes (24, 56).

We have shown, in agreement with others (45), that levels of actin mRNA do not vary between yeast and hyphal growth, using our conditions to generate yeast and hyphae. These results disagree with a recent report by Paranjape and Datta that actin mRNA levels increase during morphogenesis (38). Our conditions differed from theirs in that we used (i) yeast cells in balanced growth, rather than stationary-phase yeast cells, as an inoculum and (ii) the same temperature and media for growth of yeast and hyphal forms. The increase in actin mRNA reported by Paranjape and Datta was probably caused by a resumption in growth from stationary phase, since stationary-phase organisms do not have easily measured levels of actin mRNA (not shown). The differences in yeast and hyphal actin mRNA levels that they reported could have been a result of the different temperatures or media used to grow yeast and hyphal forms and not a true difference in production of actin mRNA between growth forms.

The data presented in this report strongly support the belief that a major 47,000-molecular-weight antigen which circulates in patients and is highly immunogenic is enolase. However, data from other laboratories suggest that there may be more than one *C. albicans* antigen of similar size. Using polyclonal rabbit antiserum to a fungal extract, Matthews and Burnie cloned a partial cDNA whose sequence predicted a protein with high homology to a portion of the *S. cerevisiae* gene for HSP90 (31). A recombinant fusion pro-

tein encoded by this clone bound antibodies which reacted in immunoblots with *C. albicans* proteins of molecular weights 47,000 and 92,000, supporting their hypothesis that a 47,000-molecular-weight component is a breakdown product of a larger heat shock protein.

Evidence is accumulating that *C. albicans* enolase may be important as a marker of disseminated *C. albicans* infections. Buckley and coworkers found antigenic cross-reactivity between *S. cerevisiae* enolase and a *C. albicans* major 48,000-molecular-weight antigen which circulates and is highly immunogenic in patients with disseminated candidiasis (30, 51). However, no data on the sequence or enzyme activity of the antigen have been published. The reaction of the fusion protein with the monoclonal antibody used to measure the antigen confirms that enolase is a major circulating antigen in patients with disseminated candidiasis.

Given the usefulness of *C. albicans* enolase as a marker for disseminated candidiasis, the enzyme's location within the fungal cell and its ability to stimulate host immune responses are of interest. The lack of reactivity of whole cells with antiserum to the fusion protein in immunofluorescence, along with the inability to detect enolase enzymatic activity by using whole cells, indicates that enolase is not present on the surface of *C. albicans*. In contrast to surface polymannose epitopes, which stimulate antibody responses during colonization, production of antienolase antibodies apparently occurs only during infection (51). Perhaps large numbers of fungi or damaged fungi are needed before enolase is released from fungal cells in quantities large enough to mount an immune response. We detected enolase protein by immunoblotting and enolase enzymatic activity in yeast and hyphal whole cell extracts prepared by breaking cells with glass beads (not shown). The presence of enolase in the Zymolyase cell wall digests, as shown in the radioimmunoprecipitation experiment, could have been the result of cell lysis or the presence of enolase within the cell wall. However, there are examples of surface locations and nonglycolytic functions for glycolytic enzymes in other organisms. Glyceraldehyde 3-phosphate dehydrogenase has been found on the surface of schistosomes, where it has been postulated to be associated with resistance to infection (15), and on streptococci (28). In fungal infections, the abundant immunostimulatory glycolytic enzymes are markers of infection which will be valuable diagnostically and may be important pathogenically.

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