

Immunologic Recognition of a 25-Amino Acid Repeat Arrayed in Tandem on a Major Antigen of *Blastomyces dermatitidis*

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Abstract

A 120-kD glycoprotein antigen abundantly expressed on *Blastomyces dermatitidis* yeasts is a target of cellular and humoral immune responses in human infection. To investigate the antigen and immune response more carefully at the molecular level, we screened an expression library from *B. dermatitidis* to identify clones that encode this antigen, designated WI-1. A 942-bp cDNA was isolated by immunologic screening with polyclonal, rabbit anti-WI-1 antiserum. Northern hybridization analysis showed that the cDNA hybridized to yeast message $\cong 3.9$ kb. DNA and deduced protein sequence analysis of the clone demonstrated a 25-amino acid repeat arrayed in tandem, present in 4.5 copies near the 5' end, and rich in predicted antigenic epitopes. Further analysis showed strong homology in these tandem repeats with invasins, an adhesin of *Yersinia*. Cloned cDNA was used to express a 30-kD fusion protein strongly recognized in western blots by rabbit anti-WI-1 antiserum, and by sera from all 35 blastomycosis patients studied. The fusion protein product of subcloned cDNA encoding only the tandem repeat also was strongly recognized in western blots by sera from the 35 blastomycosis patients, but not by sera from 10 histoplasmosis and 5 coccidioidomycosis patients. An antigen-inhibition radioimmunoassay showed that the tandem repeat alone completely eliminated rabbit and human anti-WI-1 antibody binding to radiolabeled native WI-1. From these results, we conclude that the 25-amino acid repeat of WI-1 displays an immunodominant B cell epitope, and that the carboxyl-terminus of the molecule exhibits an architecture that may promote adhesion of *Blastomyces* yeasts to host cells or extracellular matrix proteins and ultimately provide a clearer picture of the molecular pathogenesis of blastomycosis. (*J. Clin. Invest.* 1993. 92:330–337.) Key words: blastomycosis • tandem repeat • serodiagnosis • dimorphic fungus • cDNA

Introduction

Blastomycosis, an endemic systemic mycosis accompanied by protean manifestations involving the lung, skin, bone, genitourinary tract, and brain, results from infection with the thermal dimorphic fungus *Blastomyces dermatitidis*. Humans and

lower animals such as dogs are primarily infected by inhaling aerosolized conidia from soil, where the organism dwells as a saprophyte. At body temperature, the conidia convert to the parasitic or invasive yeast forms that cause disease.

A lack of understanding of the antigens of *B. dermatitidis* yeasts has hampered the development of reliable assays for diagnosis and study of immunologic features of blastomycosis. We recently described a novel 120-kD surface protein abundantly expressed on *B. dermatitidis* yeasts (1) and have demonstrated that the antigen is a target of antibody (1) and cell-mediated immune responses in infected humans (2). Based on these observations, we developed a radioimmunoassay to reliably diagnose infection by detecting antibody against the protein and have characterized T cells directed against processed forms of the protein in the course of human disease. Results from these preliminary studies indicate that the antigen holds considerable promise for improved diagnosis and study of the immunologic features of blastomycosis.

In our initial work with the 120-kD antigen, which we have designated WI-1, we employed crude techniques to isolate small amounts of partially purified protein and provided limited characterization of the molecule. In the present paper, we report the cloning of cDNA encoding a portion of WI-1. In addition to providing an abundant source of recombinant antigen and detailed structural information about the molecule, our work demonstrates that the carboxyl-terminus of WI-1 encompasses antigen-rich, tandem repeats and an immunodominant epitope against which human B cell responses are directed.

Methods

Fungi. American Type Culture Collection (ATCC) strains 60636 and 26199 were used for these studies (ATCC, Rockville, MD). These represent virulent isolates that have been associated with human disease. Stock cultures were maintained in the yeast form on 7H10 agar enriched with oleic acid-albumin complex (Sigma Chemical Co., St. Louis, MO) at 37°C. Yeasts were grown in Erlenmeyer flasks containing brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37°C in a gyrator shaker at 120 rpm for 72 h. Cells were harvested by filtration through a sintered glass filter and washed with saline.

Extraction of RNA and synthesis of cDNA. Total RNA was extracted from freshly grown *B. dermatitidis* yeasts using a phenol-chloroform technique (3). Diethylpyrocatechol (DEPC)-treated water and RNase-free glassware and plasticware were used for extracting, purifying, and analyzing RNA. A 10-g wet-wt pellet of yeasts was suspended in 10 ml lysis buffer (0.1 M Tris, 0.1 M lithium chloride, 0.01 M dithiothreitol, pH 7.0). The cell suspension was transferred to a prechilled metal cannister containing 10 ml 0.45–0.50-mm glass beads, 15 ml phenol-chloroform-isoamyl alcohol (24:24:1), and 2 ml 10% SDS. The cannister was placed into an MSK homogenizer (Braun Biotech Intl., Allentown, PA) fitted with a carbon dioxide cooling device and agitated at 2,000 strokes/min for 30 s intervals before cooling. Nearly complete disruption of cells was accomplished after five intervals. The chilled

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mixture was centrifuged at 5,000 g for 15 min at 4°C. The aqueous layer of each tube was extracted twice with phenol and then repeatedly with phenol-chloroform-isoamyl alcohol to clear the interface of precipitate. Nucleic acid was precipitated with 100% ethanol, rinsed with 70% ethanol, dried, and diluted and stored in DEPC-treated water at -70°C. Poly (A)⁺ mRNA was isolated from whole cell RNA with oligo (dT) cellulose affinity columns (Boehringer Mannheim Biochemicals, Indianapolis, IN) using previously described methods (4). Composition of whole cell RNA and poly (A)⁺ mRNA preparations was followed by agarose gel electrophoresis and OD_{260:280} was used to estimate RNA concentration and purity. A 20-g wet-wt pellet of *B. dermatitidis* yeasts strain ATCC 60636 yielded 50 mg total RNA with OD_{260:280} ratio of 2.17. Affinity purification of 10 mg total RNA yielded ~100 µg poly (A)⁺ mRNA. Gel electrophoresis of total RNA showed abundant and distinct 28S and 18S ribosomal bands. Poly (A)⁺ mRNA showed little remaining ribosomal RNA and a size range up to ~14 kb.

Starting with 5 µg poly (A)⁺ RNA from strain 60636, first- and second-strand cDNA were synthesized using murine maloney leukemia virus reverse-transcriptase, oligo (dT) primer, and other reagents and methods specified in a commercial synthesis kit (Pharmacia LKB Biotechnology, Piscataway, NJ). The ends of double-stranded cDNA were filled in with Klenow fragment and ligated to EcoRI/NotI adaptors with the sequence d(5'-AATTCGCGGCCGC-3'). Sephacryl S-400 spun columns (Pharmacia LKB Biotechnology) were used to purify cDNA fragments > 400 bp. Each aliquot of cDNA was ligated into 100 ng of a dephosphorylated phagemid vector, pBluescript II (KS⁺) (Stratagene, La Jolla, CA), which contains transcriptional and translational start sequences from the *lac Z* gene. After ligation, cDNA was electroporated into competent *Escherichia coli* XL1-Blue (Stratagene), which were prepared as described (5).

Screening the cDNA expression library with antibody and colony hybridization. Transformed *E. coli* were plated on LB agar in 150-mm-diameter petri plates (Falcon Plastics, Cockeysville, MD). After overnight growth at 37°C, bacteria were replica-plated onto a fresh agar plate, and onto a 150-mm nitrocellulose sheet (Schleicher & Schuell, Inc., Keene, NH). After colonies grew to 1–2 mm on nitrocellulose, the filter was transferred to a fresh LB agar plate supplemented with 5 mM isopropylthiogalactoside (IPTG)¹ and incubated for an additional 2–4 hours. Bacteria on the filters were lysed and the filters processed for immunoscreening as described (6). Rabbit antiserum specific for WI-1 (anti-WI-1 titer, 1:10,240, as measured by radioimmunoassay [1]) was used to screen a portion of the expression library. The serum was preabsorbed with *E. coli* lysate (Promega Biotec, Madison, WI) overnight at 4°C. Preliminary experiments determined that preabsorbed antiserum diluted 1:2,000 could discriminate 50 ng WI-1 spotted onto nitrocellulose from background staining of lysed *E. coli* proteins on the filter. Immunoscreeing was performed with this antiserum according to previously described methods (6). Rabbit anti-WI-1 bound to nitrocellulose was detected with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Promega Biotec). Positive colonies were isolated from original replica plates, replated, and rescreened with antibody until they were purified.

A 942 bp WI-1-cDNA identified through immunoscreening was used subsequently to screen the library by colony hybridization to attempt to identify additional, longer cDNAs. The entire 942-bp fragment was removed from the vector by digestion with NotI (Bethesda Research Laboratory, Gaithersburg, MD), and purified from low melting point agarose (Bethesda Research Laboratory) using GeneClean (BIO 101, Inc., La Jolla, CA). A 100-ng aliquot of the fragment was radiolabeled with [α -³²P]dCTP to a specific activity of ~10⁹ cpm/µg using random oligonucleotides as primers (IBI-Kodak Co. New Haven, CT). Colony hybridization was performed as described (6). Positive colonies were isolated from original replica plates, replated, and rescreened with probe until they were purified.

Northern hybridizations. Total RNA samples (20 µg) were electro-

phoresed through 1% agarose gels in MOPS/2.2 M formaldehyde for 16–18 h at 30 V and transferred by capillary action to Nytran (Schleicher & Schuell, Inc.) membranes using 10× SSC. DNA fragments for hybridization were radioactively labeled with [α -³²P]dCTP using random oligonucleotides as primers (IBI-Kodak Co.). WI-1 cDNA probe was obtained as described for colony hybridization. An actin DNA probe from *Histoplasma capsulatum* was the generous gift of E. Keith and was used to assess loading variation. RNA molecular weight markers were purchased from BRL-Gibco. Probes were hybridized to filters in 40% formamide for high stringency or 20% formamide for low stringency, 0.25 M Na⁺ (PO₄), 1 mM EDTA, 7% SDS at 42°C for 16–24 h. Hybridized filters were washed first in 2× SSC at room temperature for 2–5 min, then in 0.25 M Na⁺ (PO₄), 1 mM EDTA, 2% SDS at 64°C for high stringency or 55°C for low stringency twice for 10 min, next in 0.04 M Na⁺ (PO₄), 1 mM EDTA, 1% SDS at 64°C for high stringency or 55°C for low stringency once for 40–60 min, and finally rinsed in 4× SSC at room temperature (7). Washed blots were used to expose XAR-5 film, Eastman Kodak Co., Rochester, NY with intensifying screens at -80°C.

Sequence analysis. Single-stranded and double-stranded plasmid sequencing was performed according to the protocols supplied using a Sequence 2.0 polymerase kit (United States Biochem. Corp., Cleveland, OH). Single-stranded DNA was prepared from pKS⁺ phagemid clones by standard methods (8). Sequence primers homologous to the pKS⁺ vector polylinker were the T3 and SK primers (United States Biochem. Corp.). Three sets of primers homologous to internal sequences in the cDNA insert were purchased from Operon Technologies, Inc. (Alameda, CA). The complete sequence of both strands was determined. Both nucleotide sequences and the derived amino acid sequences were used to search the GeneBank and EMBL databases using programs supplied by the Genetics Computer Group (8).

Expression and immunologic assessment of fusion protein. Transformed *E. coli* was grown in LB media containing 50 µg/ml ampicillin and 12.5 µg/ml tetracycline in a gyrator shaker at 37°C. When the density of the culture reached OD₆₀₀ ≈ 0.6 (after ~4 h of growth), 5 mM IPTG was added. Optimal *lac Z* gene expression and production of the fusion protein was examined at 1-h intervals after induction. Bacteria were centrifuged at 5,000 g for 15 min at 4°C. A lysate was prepared by resuspending the pellet in 1× gel loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) equivalent to 0.1 vol of the original culture. The lysate was heated to 100°C for 3 min and centrifuged at 12,000 g for 1 min at room temperature. Lysates of *E. coli* containing vector alone to be used as antigen inhibitors of antibody binding in radioimmunoassays (RIAs) were prepared without reducing agents. Bacteria were resuspended 1:4 (wt/vol) in a lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 100 µM PMSF, and 10% sucrose) to which lysozyme (1 mg/ml) and Triton x-100 (0.1%) were added before centrifugation at 12,000 g for 30 min. Lysates were examined by SDS-polyacrylamide gel as described by Laemmli (9). Gels were stained for protein with Coomassie brilliant blue.

To assess antibody recognition of the fusion protein, western blots of gels were prepared as described (10). Human antisera used in these experiments were from blastomycosis patients in whom antibody responses to WI-1 had previously been demonstrated by RIA (1), and from seronegative control subjects with other fungal diseases or no illness. Sera had been stored at -70°C for up to 8 y prior to testing.

The fusion protein was electroeluted from polyacrylamide gels using an electrophoretic concentrator (Isco, Inc., Lincoln, NE) as described (1) for further immunologic studies. To determine the relative importance of immunologic determinants on the fusion protein, an antigen-inhibition RIA was used. It examined the ability of samples of eluted protein or crude *E. coli* lysate to block anti-WI-1 antibody binding to radiolabeled WI-1. Briefly, RIAs were constructed using 0.1–5 ng radiolabeled WI-1 as a target. Assays were done in 12 × 75 mm acid-washed glass tubes. We used 0.1% BSA in 0.04 M Na phosphate, 0.15 M NaCl, pH 7.5 (BSA-PBS) as a carrier solution. Each experimental or control sample was analyzed in duplicate or triplicate. A test sample

1. Abbreviation used in this paper: IPTG, isopropylthiogalactoside.

consisted of 0.1 ml of diluted serum and 0.1 ml of radiolabeled antigen. When an antigen-inhibition RIA was done, 0.1 ml of the inhibitor or BSA-PBS was added to diluted antibody in appropriate tubes. All tubes were incubated 4–18 h at 4°C before radiolabeled antigen was added. For each antigen-inhibition RIA performed, a preliminary RIA was done to determine the dilution of serum that bound ~ 50% of radiolabeled antigen target. This dilution of serum was added to tubes of the antigen-inhibition RIA. For RIAs and antigen-inhibition RIAs, 2 mg insoluble staphylococcal protein A (Sigma Chemical Co.) was added to each sample and control tube after overnight incubation at 4°C. Precipitates were centrifuged at 2,000 g for 10–40 min and a volume of supernatant was removed from each tube for counting in a gamma counter. We corrected for coprecipitation in RIAs by the method of Minden and Farr (11).

Results

Identification of WI-1 cDNA clones. Approximately 10^6 recombinants were obtained in the expression library. Rabbit anti-WI-1 antibody was used to screen 2,000 recombinants and identified two positive clones. The cDNA inserts in plasmids from these clones were estimated to be approximately 700 and 950 bp by NotI digestion of the plasmids; EcoRI digestion yielded a common fragment of approximately 430 bp. Colony hybridization was used to screen an additional 10,000 recombinants and identified an additional 25 independent, positive clones. NotI digestion demonstrated each cDNA to be ~ 950 bp and contain the common enzyme restriction site for EcoRI.

Northern hybridizations using WI-1 cDNA to probe *B. dermatitidis* RNA illustrate that the full-length message for WI-1 is ~ 3.9 kb (Fig. 1). WI-1 message is abundantly expressed in the yeast strain 60636, from which the partial cDNA was cloned, as well as in the unrelated strain 26199 (data not shown).

WI-1 cDNA sequence. DNA sequence analysis of the largest clone isolated by immunoscreening is shown in Fig. 2. The 942-bp sequence contains two open reading frames. One open reading frame predicts an amino acid sequence conforming very closely with the amino acid composition of WI-1 in containing large amounts of cysteine, tyrosine, and proline (Klein, B., manuscript in preparation). The predicted molecular mass of the recombinant antigen is 25.5 kD; 3.5 kD encoded by

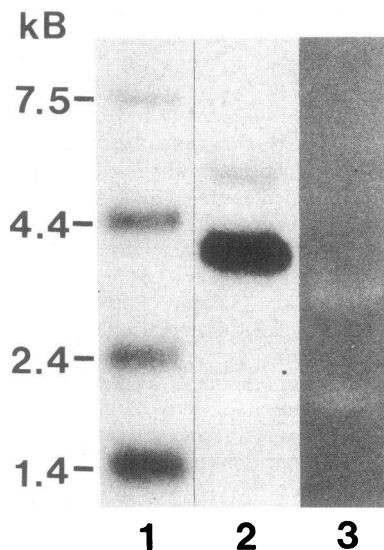


Figure 1. WI-1 gene expression in *Blastomyces dermatitidis* yeasts. Northern hybridization analysis of total RNA (20 µg) from ATCC strain 60636 probed with a 942-bp WI-1 cDNA fragment (lane 2). Ethidium bromide staining of gel prior to transfer shows 28S and 18S ribosomal bands (lane 3). Molecular size standards are shown in lane 1.

GGATCCATATCATGAGAAGTATGATTGGGATCTCTGGAATAAGTGGTCAACAAGGACCC	60
D P Y H E K Y D W D L W N K W C N K D P	20
Invasin-	
CTACAAGTGGGACTGGGACCCATCTCATGAGAAGTATGATTGGGATCTCTGGAATAAGTGG	120
Y N C D W D P S H E K Y D W D L W N K W	40
Invasin -	
GTGCAACAAGGACCCCTACAAGTGGGACTGGGACCCATATCATGAGAAGTATGATTGGGA	180
C N K D P Y N C D W D P Y H E K Y D W D	60
like	
TCTCTGGAATAAGTGGTCAACAAGGACCCCTACAAGTGGGACTGGGACCCATATCATG	240
L W N K W C N K D P Y N C D W D P Y H E	80
Invasin-like	
GAAATGATTGGGATCTCTGGAATAAGTGGTCAACAAGGACCCCTACAAGTGGGACTGG	300
K Y D W D L W N K W C N K D P Y N C D W	100
Invasin-like	
GGACCCATCTCATGAGAAGTACGATTGGGATCTCTGGAGTAAGTGGTCAACAAGGACCGA	360
D P S H E K Y D W D L W S K W C N K H D	120
CGAGCACGACAAACATCCATTGTGCGCCTGTCTGTGACCCCTCTCAGGCAAAATCACTG	420
E H D K H P L C P V C D P L S G K N H C	140
TCATCCAACTTCTCTGTGTCAGCACAGGACCCACTACCACTGCGCCTGCCGCGCTGG	480
H P T T S C V S T G H H Y H C A C R A G	160
CTACAAGGCTAGCCATTATAGCCATGACCAAGCATTTCGCGATGCCAGTCAAAGGCTA	540
Y K A S H Y S H D H K H F R M P V K G Y	180
TGAATTCCTTTGTTTCCACCGCCGATACCAAGTGAATGCTCTTTCGACGCGTTACCC	600
E F L V F T G P H T K C N V L C D G Y P	200
GCACAAGCCAGCCCATGAGCTTTGCGGCGAGGTTAAGGTTCAATATTTCGCGGCCATG	660
H K P A H E L C G E V K V H N Y C G P	219
AGCTTCCTTTGAGGAAAGCGCAGAAAGAGGAAATTTGGGGTATTTTGGAGTACTTGA	720
ACAGATTTATTTTCCTCGTTTTGAGGGAGGAGAAAGCGAACATGTAATTTTGTAGATGG	780
GTGGAAGGCTTCATCTGACCTCTGGACGAGGTCAATTTTCCCTTTTATTATGGATAAAT	840
ATTGATGAAGTTGGATAATAGGAGACATCTTTTGCATAATTAATAGTTATATATATGA	900
GTTAGATTACCTTCTTAATTAGATGCTTTAATGATTTTGTG	942

Figure 2. Nucleotide and predicted protein sequence of partial cDNA encoding WI-1. The predicted protein sequence is indicated below the nucleotide sequence. Numbering to the right indicates the nucleotide and protein positions. The 25-amino acid repeats arrayed in tandem, present in 4.5 copies near the 5' end, are overlined with arrow bars. WI-1 sequence showing similarity with invasin appears shaded. The asterisk overlying nucleotide position 359 denotes the FokI restriction site used to cut and subclone cDNA encoding only the tandem repeats. A polyadenylation site, located in the 3' nontranslated region, is comprised of the tripartate structure TAGT (nucleotides 886–889), an upstream sequence TAG (859–861) and a downstream sequence TTT (927–929). This is similar to the polyadenylation site described for the CYC1 gene locus in *Saccharomyces cerevisiae* (29).

plasmid β -galactosidase fusion sequences and the remainder by the WI-1 insert. The DNA sequence predicts an amino acid sequence comprising a 25-amino acid repeat arrayed in tandem, present in 4.5 copies near the 5' end, with amino acid variations in only two positions (serine vs. tyrosine, residues 3,28,53,78,103; and serine for asparagine, residue 113).

DNA and deduced amino acid sequences of WI-1 showed similarity to other sequences in the GeneBank database (Fig. 2 and 3). 17 amino acid residues of WI-1 spanning the end of one tandem repeat and the beginning of another showed 35% identity and 88% homology to the protein adhesion of *Yersinia*, invasin (12). 36 amino acid residues outside the tandem repeat of WI-1 (spanning nucleotides 391 to 498) comprise a cysteine-rich region showing 27% to 41% identity and a high degree of similarity to an EGF-like domain expressed on numerous pro-

A

WI-1	LWNKWCNKDPYNCWDWP
Invasin	LWGEWGS LTAYS SDWQS

B

WI-1	CDPLSGKN-HCHPTTSCVST
EGF Consensus	C-L-NNGGCSQX-XCXNT
	GHHYHCACRAGYKASHY....
	XGGYXCXCEGYXLXDGKXCX

Figure 3. Homology of WI-1 sequence with other sequences in the GenBank and EMBL data bases. In *A* and *B*, sequence identity is indicated by a vertical line. A conservative amino acid substitution is indicated by a colon.

Asterisks corresponding to EGF consensus sequence denote the presence and spacing of cysteine residues, a prominent feature of the motif.

teins that confer adhesion to cells or extracellular matrix (13). This similarity with EGF-like domains expressed in at least seven other proteins is not likely due to chance alone (*P* values ranging from $< 1.7 \times 10^{-8}$ to < 0.01), according to a recently described algorithm (14).

The predicted structure and antigenicity of the protein encoded by the WI-1 insert was analyzed using algorithms of Chou and Fasman (15) and Kyte and Doolittle (16). The protein, which corresponds to the carboxyl terminus, is hydrophilic along most of its length except in the portion showing an EGF-like domain, in which the molecule exhibits a hydropho-

bic region. Residues in this area are predicted to form a beta-pleated sheet, a common structural motif of EGF-like domains based on conserved sequence and dictated by the position of the disulfide bonds (13). In contrast, residues in each region showing similarity with invasin are predicted to form several turns and an α helix at the initiation. The 25-amino acid repeat arrayed in tandem folds back on itself several times, and these repeats, which also encompass the invasin-like domains, are packed with predicted antigenic sites.

Expression of the fusion protein. Lysates of *E. coli* XL1-blue transformed with the pBluescript II vector alone or the vector plus 942-bp WI-1 insert, studied by SDS-PAGE, show that a fusion protein of ~ 30 kD is expressed by 2 h and produced maximally by 4 h after induction with IPTG (Fig. 4 *A*, lane 2, see arrow). This protein may represent WI-1 cleaved from the 3.5 kD β -galactosidase fusion partner, since the same lysate also contains smaller amounts of 33.5 kD protein that is enriched in comparison to control lysates. Rabbit anti-WI-1 specifically recognizes both of these proteins in a western blot (Fig. 4 *B*).

Human immune responses to WI-1 fusion proteins. 46 sera from 35 patients with culture-confirmed cases of blastomycosis were tested for recognition of proteins in the *E. coli* lysates. 19 (54%) of the patients had isolated pulmonary disease, the remainder had disseminated disease involving skin alone (8 patients); lung and skin (5); lung, skin, and brain (1); brain alone (1); and eye alone (1). Titers of antibody to WI-1 in these patients ranged from 1:100 to 1:34,000 (geometric mean titer, 1:850). All sera tested reacted specifically with WI-1 fusion protein in Western blots (Fig. 5). Some background reactivity with *E. coli* proteins in the lysates is seen in all lanes of the blots. Preabsorption of rabbit and human anti-WI-1 antisera with either the eluted fusion protein or with *E. coli* lysate containing the fusion protein nearly eliminated recognition of radiolabeled WI-1 in the RIA, whereas preabsorption with *E. coli* lysate without the fusion protein had no influence on WI-1 recognition (Fig. 6, *A* and *B*). The fusion protein further inhibited anti-WI-1 binding to 125 I-WI-1 in a dose-dependent manner (Fig. 6, *C*), with 2 μ g protein inhibiting 100% of binding by either rabbit or human antiserum.

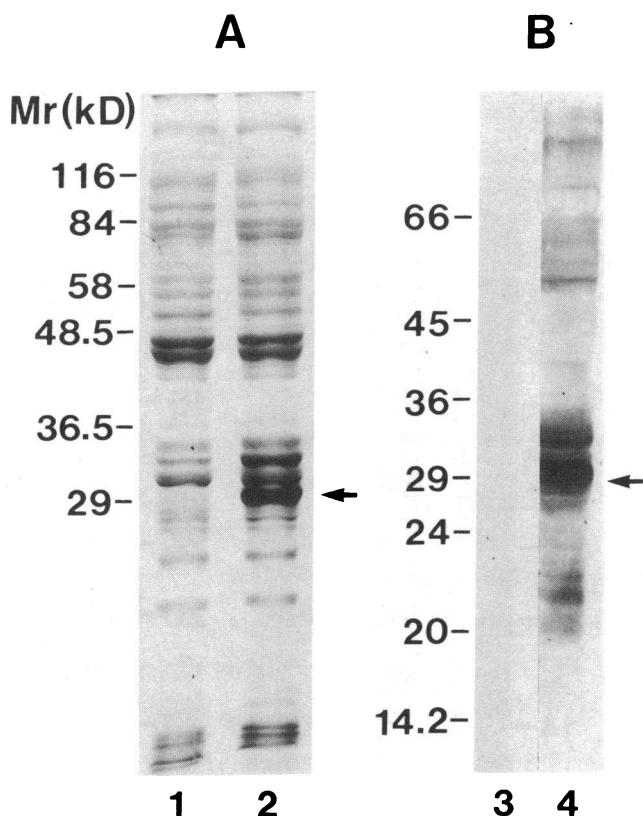


Figure 4. SDS-PAGE analysis of recombinant WI-1 protein. (*A*) 10% acrylamide gel of *E. coli* lysate containing pBluescript II plasmid alone (lane 1) or plasmid plus WI-1 cDNA insert (lane 2) 4 h after induction with IPTG. Molecular weight standards are indicated to the left of lane 1. Coomassie blue was used to stain gel. (*B*) Immunoblots of *E. coli* lysates containing plasmid alone (lane 3) or recombinant WI-1 (lane 4) probed with rabbit anti-WI-1 antiserum (RIA titer, 1:10,240) diluted 1:2,000. Molecular weight standards are indicated to the left of lane 3.

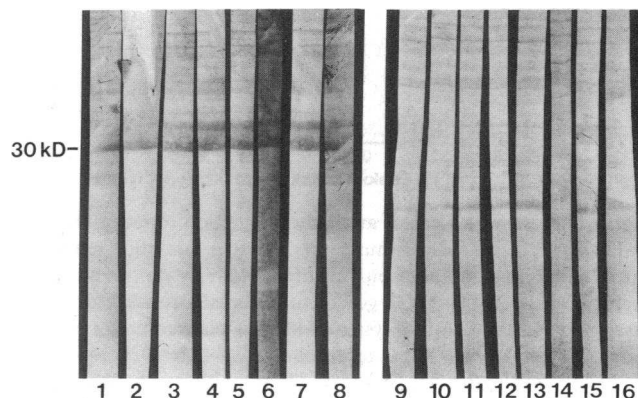


Figure 5. Antibody recognition of recombinant WI-1 in blastomycosis patients. Immunoblots of *E. coli* lysates containing recombinant WI-1 (lanes 1-8) or vector control (lanes 9-16) 4 h after induction with IPTG, probed with antisera from blastomycosis patients. Sera were diluted 1:1,000. Results are shown for 8 patients (similar results were obtained for an additional 27 blastomycosis patients).

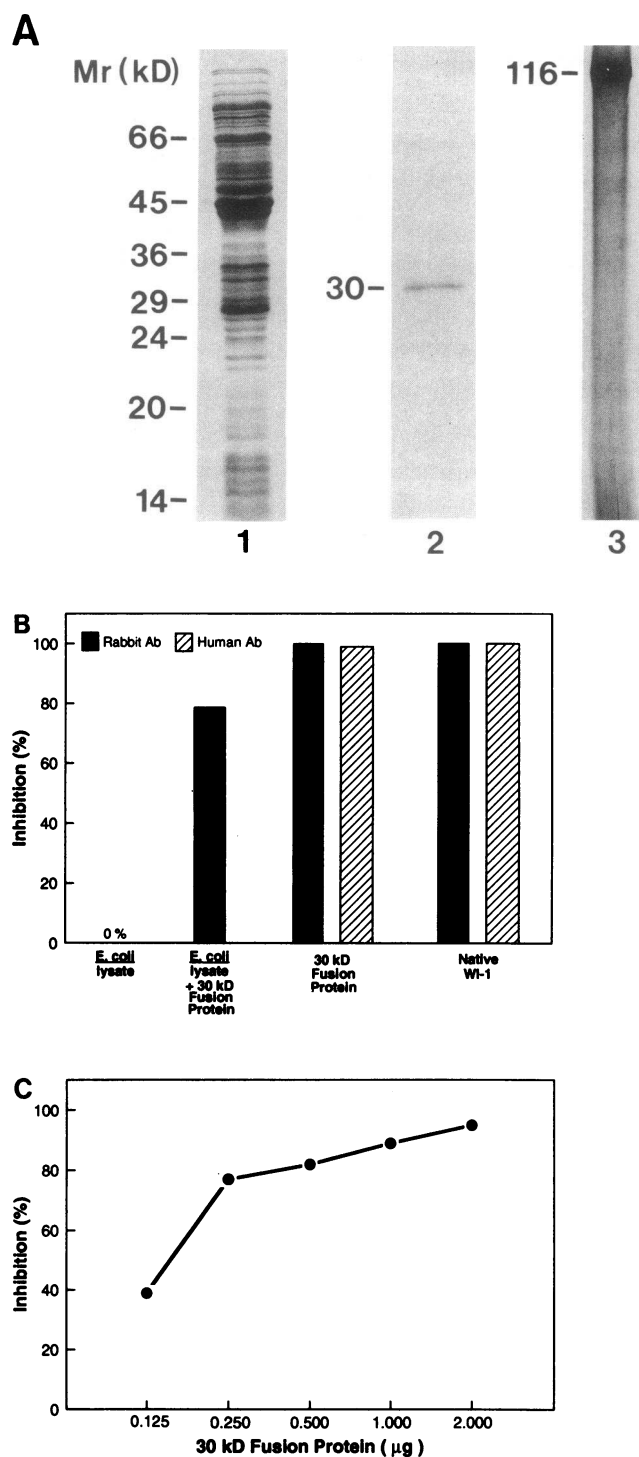


Figure 6. Human and rabbit antibody recognition of recombinant and native WI-1 by radioimmunoassay. An RIA inhibition test measured inhibition of antibody binding to ^{125}I -WI-1 by various antigen preparations. (A) SDS-PAGE gels of antigens used as inhibitors in the assay: representative *E. coli* lysate containing plasmid vector alone (lane 1), electroeluted 30-kD recombinant WI-1 (lane 2), and HPLC-purified native WI-1 (lane 3). (B) Inhibition of antibody binding to ^{125}I -WI-1 by *E. coli* lysate (50 μg protein), *E. coli* lysate "spiked" with electroeluted recombinant WI-1 (2 μg of fusion protein added to lysate), electroeluted recombinant WI-1 alone (2 μg protein), and native WI-1 (500 ng). (C) Dose-dependent inhibition of antibody binding to ^{125}I -WI-1 by electroeluted 30-kD recombinant WI-1. Dilutions of human and rabbit antisera yielding 50% radiolabeled antigen binding were used in the assays.

Subcloning and immunologic study of the tandem repeat. We examined whether the tandem repeat of cloned WI-1 cDNA was an important site for anti-WI-1 antibody recognition. Restriction sites bounding the repeats were used to subclone a 372-bp fragment encoding 4.5 copies of the tandem repeat into pBluescript II (Fig. 2). A FokI digest of WI-1 cDNA yielded a 1,678-bp fragment including the tandem repeats plus five additional bases at the 3' end and a portion of vector at the 5' end. After the ends were made blunt with Klenow treatment, a HindIII digest, which cuts in the polylinker site of pBluescript, was used to separate the cDNA and vector. The 372-bp fragment was purified and cloned directionally into pBluescript II cut with Hind III and Sma I. Ligated cDNA was electroporated into *E. coli* XL-1 Blue and the transformants were assessed for recombinants by blue/white color selection. Fusion protein encoded by subcloned cDNA was expressed in *E. coli* as above. When studied by SDS-PAGE, the fusion protein comprised by tandem repeats is produced maximally by 4 h after induction with IPTG, migrates at ~ 21 kD, and is recognized specifically by rabbit anti-WI-1 antiserum in a Western blot (Fig. 7).

Sera from the 35 blastomycosis patients studied above were examined for recognition of the tandem repeat by Western blot. All of them reacted strongly with the recombinant protein (Fig. 8). None of the sera from ten patients with histoplasmosis (complement fixation titers, 1:32 to 1:512; median titer, 1:128) or five patients with coccidioidomycosis (complement fixation titers, 1:32 to 1:64; median titer, 1:64) reacted with the recombi-

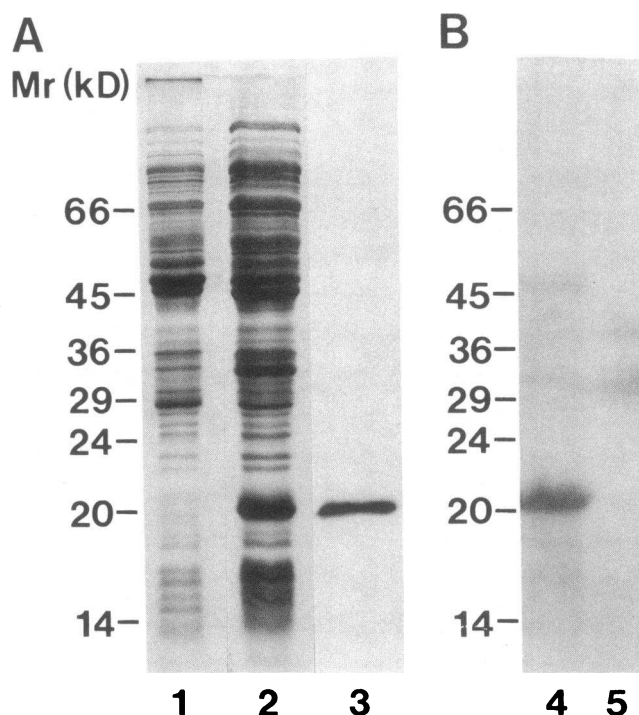


Figure 7. Fusion protein product of subcloned 372-bp WI-1 cDNA encoding tandem repeat. (A) 12.5% SDS-PAGE gel of *E. coli* lysate containing pBluescript II plasmid alone (lane 1), plasmid plus WI-1 cDNA insert (lane 2) 4 h after induction with IPTG, and electroeluted fusion protein (lane 3). Molecular weight standards are shown to the left of lane 1. Coomassie blue was used to stain gels. (B) Immunoblots of *E. coli* lysate containing recombinant WI-1 tandem repeat (lane 4) or plasmid alone (lane 5) probed with rabbit anti-WI-1 antiserum (RIA titer 1:10,240) diluted 1:2,000.

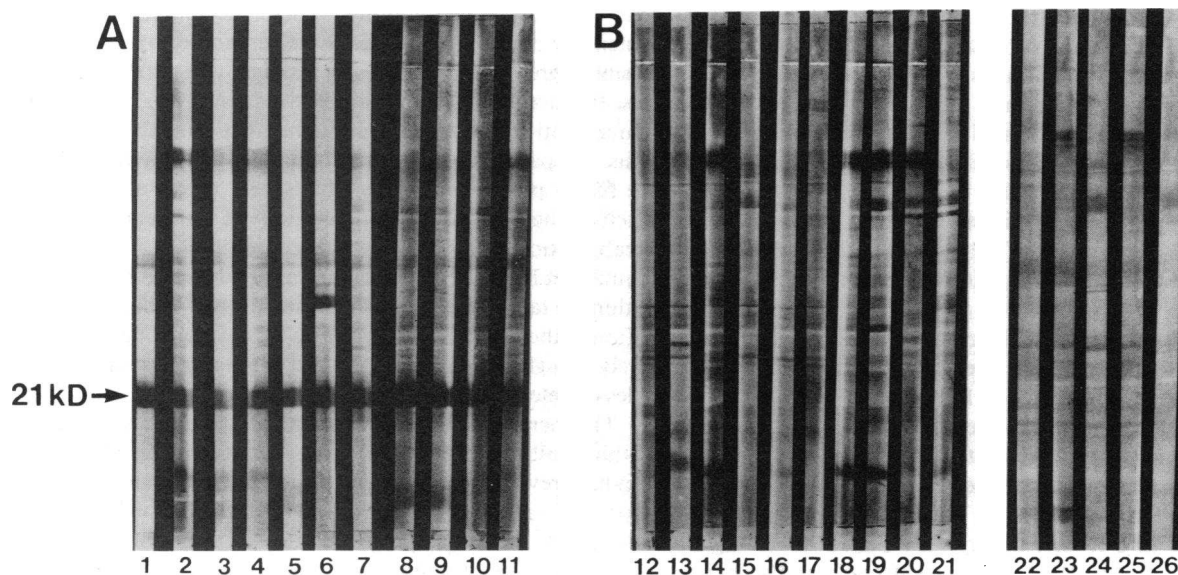


Figure 8. Antibody recognition of recombinant WI-1 tandem repeat in blastomycosis patients and control subjects. (A) Immunoblots of *E. coli* lysates containing WI-1 tandem repeat (lanes 1–11) probed with antisera from blastomycosis patients. Sera were diluted 1:50. Results are shown for 11 representative patients (similar results were obtained for an additional 24 patients). (B) Immunoblots of *E. coli* lysates containing WI-1 tandem repeat probed with antisera from 10 histoplasmosis patients (lanes 12–21) and five coccidioidomycosis patients (lanes 22 to 26). Sera were diluted 1:50.

nant protein, although some background reactivity with *E. coli* proteins in the lysates is evident (Fig. 8).

To assess the relative importance of the tandem repeat in antibody recognition of WI-1, we tested the 21-kD fusion protein in the antigen-inhibition RIA using anti-WI-1 antisera from ten blastomycosis patients and an immunized rabbit. The tandem repeat inhibited a mean of 96% (range 85–100%) of the antibody binding to radiolabeled WI-1 in the blastomycosis patients, and 87% of the binding of antibody in the immune rabbit (Fig. 9). The results were uninfluenced by whether the

fusion protein was tested after purification by elution, or in the presence of *E. coli* lysate, whereas *E. coli* lysate alone without the fusion protein had no influence whatsoever on anti-WI-1 antibody binding of radiolabeled WI-1.

Discussion

The diagnosis and basic investigation of blastomycosis has been seriously hampered by the lack of well-defined antigens. We recently described a novel antigen, WI-1, a 120-kD glycoprotein that is abundantly displayed on the surface of *B. der-*

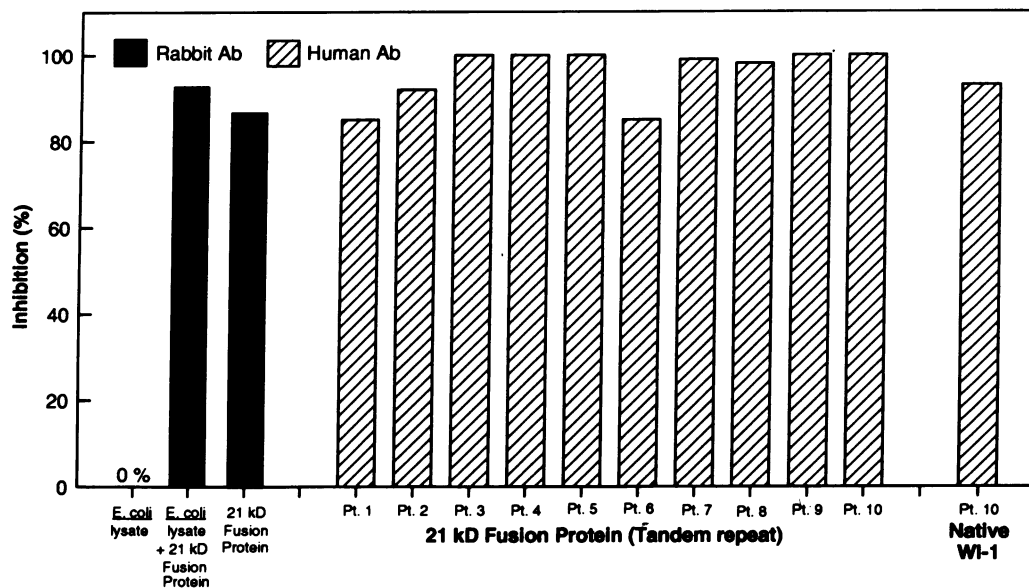


Figure 9. Human and rabbit antibody recognition of recombinant WI-1 tandem repeat by radioimmunoassay. An RIA inhibition test measured inhibition of antibody binding to ^{125}I -WI-1 by various antigen preparations. Antigens used as inhibitors were: crude *E. coli* lysate containing plasmid vector alone (see Fig. 7, lane 1), electroeluted recombinant WI-1 tandem repeat (see Fig. 8, lane 3), the lysate and eluted protein preparations combined, and HPLC-purified native WI-1 (see Fig. 7, A, lane 3). Protein content of inhibitors were 50 μg crude *E. coli* lysate, 2 μg recombinant WI-1 tandem repeat, and 500 ng native WI-1. Dilutions of rabbit and human antisera yielding 50% radiolabeled antigen binding were used in the assay.

matitidis yeasts (1), shed into the medium of actively growing cells (Klein, B., manuscript submitted for publication) and highly conserved in wild-type strains (1). The interaction of *B. dermatitidis* yeasts with the human immune system elicits a vigorous humoral and cell-mediated response directed against WI-1 and its epitopes during acquisition of resistance to infection (1, 2). Because of the possible importance of WI-1 with respect to improved serodiagnosis, study of host-pathogen interactions, and understanding of the biology of the fungus, we have sought to learn more about this antigen at the molecular level.

In this report, we describe the cloning of cDNA that encodes a portion of WI-1. Several lines of evidence support this conclusion. First, rabbit anti-WI-1 antiserum was used to screen an expression library for clones that produce immunoreactive protein, and the cDNA isolated and characterized produces a fusion protein that reacts specifically and strongly with rabbit antiserum in both a Western blot and radioimmunoassay. Second, antisera from blastomycosis patients with high antibody titers to native WI-1 also react strongly and specifically with the fusion protein in both of these assays, whereas sera from 15 histoplasmosis or coccidioidomycosis control subjects do not. Although the number of controls studied here is limited, the specificity is consistent with previous work, in which only two (3%) of 73 control subjects with other fungal infections had detectable antibody to radiolabeled, native WI-1 (1). Third, the results of analysis of the DNA sequence and deduced amino acid sequence of the cDNA are remarkably consistent with previous analysis of the amino acid composition of native WI-1, such as the high content of aromatic residues and especially the unusually large amount of cysteine (Klein, B., manuscript in preparation). Finally, northern hybridization using the cloned cDNA probe identifies a message ~ 3.9 kb, approximately the size predicted for native WI-1. Despite these lines of evidence, formal proof that we have isolated WI-1 cDNA awaits a comparison of the DNA sequence with amino acid sequence from native WI-1. In view of this fact, it may be appropriate to refer to the cDNA and recombinant protein we describe as a WI-1-like antigen sequence.

The cDNA clone studied encodes only a portion of WI-1. The uniformity in size of about 1 kb for nearly all the independently derived cDNA clones is quite striking, and suggests that RNA secondary structure may interfere with transcription beyond this point. This conclusion is supported by previous difficulties we have had with in vitro translation of heat-denatured *Blastomyces* total or poly (A)⁺ mRNA: Methyl mercury hydroxide denaturation of RNA secondary structure was required to produce large (although still incomplete) translation products (Klein, B., and J. Jones, manuscript submitted for publication). Secondary structure commonly interferes with the cloning of full-length cDNAs (6, 17). Preliminary evidence has indicated that we can use a custom primer derived from the WI-1 cDNA sequence, rather than oligo (dT)₁₂₋₁₈, to transcribe beyond the presumed, problematic area of secondary structure and isolate larger cDNAs.

The truncated form of recombinant WI-1 produced by the cloned cDNA appears to be highly antigenic and to contain an immunodominant region for antibody recognition by infected humans. The fusion protein contains 219 amino acids at the carboxy terminus of WI-1. Chou-Fasman predictions based on the deduced amino acid sequence of the fusion protein (15) indicate that the 25-amino acid tandem repeat is rich in anti-

gens, probably B cell epitopes. These predictions are based principally on the hydrophilic character, and exposed and accessible highly charged and polar residues, that appear to comprise the turns and α helices within this repeat. Our results with human anti-WI-1 antisera are consistent with these predictions. All patients tested strongly and specifically recognized the fusion protein in Western blots, and nearly all antibody reactivity against WI-1 could be removed from antiserum by preabsorption with the fusion protein in the RIA. Our experimental results further delineate the antibody recognition site to within the tandem repeat of the fusion protein. Precise identification of the residues that comprise the epitope should be relatively straightforward with the preparation of synthetic peptides or deletion mutants of WI-1 cDNA.

The theme of immunodominant B cell epitopes contained within multiple repeats arrayed in tandem on microbial antigen has previously been described in a conserved antigenic epitope of *Trypanosoma cruzi* (18) and in the circumsporozoite antigen of *Plasmodium falciparum* (19). The importance of the strong immune response directed against these determinants remains unclear. Nonetheless, identification of the immunologic importance of the tandem repeat on WI-1 will permit improved serodiagnosis of blastomycosis. This issue notwithstanding, it is well accepted that antigen-specific T cells confer acquired resistance and protect against a lethal challenge of *Blastomyces* in animal models (20). We have recently shown that acquired resistance in human infection is associated with development of WI-1-responsive T cells. Further study of the tandem repeat and the products of larger or full-length cDNAs of WI-1 will permit identification of T cell epitopes.

An examination of the DNA and deduced amino acid sequence of WI-1 offers potentially informative insights into an abundantly expressed, highly conserved molecule of *Blastomyces* whose function is unknown. The deduced amino acid sequence exhibits an interesting protein mosaic architecture with tandem interaction domains containing regions of great similarity with invasin, a 103-kD adhesin of *Yersinia*, adjacent to an EGF-like domain, a motif exhibited by numerous molecular species that mediate adhesive interactions with cells or extracellular matrix proteins. Invasin- and EGF-like domains have been extensively investigated. Invasin, a product of the INV locus, is a protein virulence determinant of *Yersinia* that promotes adhesion of the bacterium and penetration into mammalian phagocytic and nonphagocytic cells (21). Several receptors that bind invasin have been identified and each is a member of the VLA family of integrin cell adhesion molecules that expresses a common β_1 chain (22). The carboxy-terminal 192 amino acids of invasin contain the integrin binding domain (23), even though this region does not contain the tripeptide sequence Arg-Gly-Asp (RGD), a common motif for the binding site of ligands with β_1 integrins (24). Invasin and fibronectin bind to the same, or closely located sites on $\alpha_5\beta_1$ integrins, although binding by fibronectin is mediated by the tripeptide sequence RGD, whereas invasin binds with much higher affinity than does fibronectin (25). The deduced sequence of WI-1 cDNA predicts four 17-amino acid repeats in tandem that exhibit 88% homology with a portion of invasin in the 192 amino acids of the integrin-binding domain. The tripeptide sequence RGD is similarly not present in WI-1 cDNA. It remains possible that WI-1 is homologous to invasin in a region that is completely unrelated to integrin binding. However, the hydrophilic, highly exposed α helices that turn back

and forth on themselves predicted in the invasin-like domains of WI-1 cDNA would make this region of the protein suitable for ligand-receptor interactions.

The EGF-like domain in WI-1 cDNA consists of a single-copy homologue of the EGF repeat unit, which preserves many of the Cys or Gly residues characteristic of the structure, and particularly the presence of two antiparallel β -strands between Cys 3 and Cys 4 believed to comprise the EGF receptor recognition site (13). We recognize that only five of six consensus cysteines in this motif are apparent in WI-1 and that the alignment of these conserved residues is not precise when compared to the EGF consensus sequence. However, the spacing of conserved residues typically varies in EGF-like domains so that at least three types of consensus sequences have been described (13). When compared to other molecules containing EGF-like domains, WI-1 shows the best fit with the repeats of lymph node homing receptor (26), uromodulin (27), nidogen (28), and fibulin, each of which governs diverse cell-cell or cell-matrix interactions. This observation, taken together with the close proximity of the EGF repeat and multiple invasin-like domains, would support the notion that the carboxy terminus of surface exposed WI-1 may promote adhesion of *Blastomyces* yeasts to host cells or extracellular matrix proteins. Preliminary experiments indeed have shown that WI-1 functions as an adhesion-promoting protein and mediates the binding of yeasts to mammalian cells. Study and further clarification of the role of WI-1 in these interactive events may provide a clearer picture of the molecular pathogenesis of blastomycosis.

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