Genomic Cloning, Characterization, and Functional Analysis of the Major Surface Adhesin WI-1 on Blastomyces dermatitidis Yeasts*

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**WI-1 is a 120-kDa surface protein adhesin on Blastomyces dermatitidis yeasts that binds CD18 and CD14 receptors on human macrophages.** We isolated and analyzed a clone of genomic WI-1 to characterize this key adherence mechanism of the yeast. The 9.3-kilobase insert contains an open reading frame of 3438 nucleotides and no introns. The amino acid sequence of native WI-1 matches the deduced sequence of genomic WI-1 at positions 757–769, 901–913, and 1119–1138, demonstrating the cloned gene is authentic WI-1. The complete coding sequence has 30 highly conserved repeats of 24 amino acids arrayed in tandem in two noncontiguous regions of the protein. The repeat sequence is homologous to the Yersinia adhesin invasin, the C terminus displays an epidermal growth factor-like domain, and the N terminus has a short hydrophobic sequence that may be a membrane-spanning domain. The tandem repeats are predicted to be at the exposed surface of the protein, thereby explaining the adhesive properties of WI-1. The WI-1 promoter contains a CAAT box (nucleotide positions 2287–2290), TATA box (2380–2385), and CT motif (2399–2508). Transcription is initiated within the CT motif at nucleotide 2431. A 5.5-kilobase subclone containing the full coding sequence of WI-1 was expressed as a histidine-tagged fusion protein in Escherichia coli. Recombinant WI-1 has the expected molecular mass of 120 kDa, is strongly recognized in Western blots by rabbit anti-WI-1 antiserum, and binds human macrophage receptors in the same manner as native WI-1. This work clarifies a key adherence mechanism of B. dermatitidis and will permit further analysis of WI-1-mediated attachment to host cells, receptors, and extracellular matrix.

Medically important fungi pose a serious health hazard, especially for the growing numbers of immunocompromised patients worldwide (1). At the same time, there are few antifungal drugs, and little is known about pathogenic mechanisms that might serve as new drug or vaccine targets in the fungi. To establish themselves in a host, pathogens must be able to adhere to tissues. Characterization of adherence promoting molecules, or adhesins, has therefore yielded insights into a key mechanism of microbial invasion of the host. Several adhesins of the opportunistic fungus Candida albicans regulate attachment, invasion, and dissemination of the fungus and help explain the molecular pathogenesis of disseminated candidiasis (2–4). However, adhesins of other medically important fungi have not been characterized.

Blastomyces dermatitidis is a dimorphic fungal pathogen that infects the host through inhalation of conidia (5). Upon transformation into the pathogenic yeast phase, B. dermatitidis multiplies within the lung and disseminates via the blood stream and lymphatics to cause disease in the skin, bone, genitourinary tract, and brain (5, 6). Inflammatory reactions occur at the initial site of infection and at these metastatic foci. Cellular immunity is the major protective response of the host in preventing progressive disease in blastomycosis (7–10). The interaction of B. dermatitidis yeasts with host macrophages is therefore a critical event in the pathological process.

**WI-1 is a 120-kDa surface protein on B. dermatitidis yeasts (11) and the major target antigen of cellular (12) and humoral (13) immunity in infected humans.** Analysis of a partial cDNA from cloned WI-1 showed 4.5 copies of a 25-amino acid repeat arrayed in tandem near the C terminus. The repeat sequence is similar to invasin, an adhesion-promoting protein on Yersinia (14). Binding studies demonstrated WI-1 is an adhesin, which mediates attachment of yeasts to macrophages; the 25-amino acid repeat interacts with both CR3 and CD14 receptors (15).

In view of the importance of adherence in understanding and intervening in the pathogenesis of invasive fungal diseases, and because of the key role of WI-1 in binding yeasts to host tissues, we sought to characterize WI-1 more carefully at the molecular level. We describe here the complete cloning of genomic WI-1 and express and functionally analyze the adhesive properties of mature recombinant WI-1. We show the striking finding that the full-length gene contains 30 copies of the invasin-like repeat within the coding sequence. This information sheds new light on a key adherence mechanism in this pathogenic fungus.

**MATERIALS AND METHODS**

Fungal Strains—American Type Culture Collection (ATCC) strains 26199 and 60636 were used for these studies (ATCC, Rockville, MD). These strains are virulent isolates that have been associated with human disease. Strain 26199 also is the parental isolate of a collection of genetically related strains of B. dermatitidis that differ in virulence in a murine model of blastomycosis (16). Stock cultures of the strains were maintained in the yeast form on 7H10 agar enriched with oleic acid-albumin complex (Sigma) at 37 °C. Yeasts were grown in Erlenmey er flasks containing brain heart infusion 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 and Analysis of Genomic DNA—High molecular weight genomic DNA was extracted from spheroplasted yeast cells of ATCC.
strain 26199. Cells from a 50-ml culture were centrifuged for 5 min at 1000 x g, resuspended in 5 ml of 5 mM EDTA containing 10 ml of 2-mercaptoethanol, and incubated while rocking for 30 min at room temperature. The suspension was then centrifuged for 5 min at 1000 x g, and pelleted cells were suspended in 1.25 ml of a buffer containing 1 x sorbitol, 100 mM EDTA, 50 mM sodium citrate at pH 5.7, 2.5 mg/ml of lysylchymotrypsin, and 3% (w/v) poly(A) RNA. After incubation for 18 h at 37 °C, the treated cells were centrifuged for 6 min at 3000 rpm, washed three times with 1 x sorbitol, resuspended in 0.5 ml of Tris-EDTA containing 1% SDS, and heated for 20 min at 65 °C. After 0.1 ml of 5% potassium acetate was added to the cell suspension, they were incubated on ice for an additional 30 min, centrifuged for 5 min at 12,000 x g, and one volume of chloroform and once with chloroform alone. The genomic DNA was precipitated with ethanol, and resuspended in Tris-EDTA containing RNase A (20 mg/ml).

Genomic DNA was resolved over 1 h at 100 V on a 1% agarose gel and stained with ethidium bromide. For Southern analysis, genomic DNA digested with restriction enzyme was resolved overnight at 30 V on a 1% agarose gel, transferred to nitrocellulose, and probed using previously described methods (17). A WI-1 DNA designated pI.1 and described under “Results” was prepared as a purified plasmid expression vector (QIAGEN, Chatsworth, CA), which creates a fusion protein by placing 6 histidine residues at the N terminus of recombinant proteins and permits purification on a nickel affinity column (23). Genomic WI-1 was digested using BspI (Life Technologies, Inc.), and the resulting 5.5-kb fragment was treated with Klenow fragment (Life Technologies, Inc.) to create blunt ends and then purified using Gene clean (Bio 101, La Jolla, CA) after separation in low melting point agarose. The purified 5.5-kb fragment was ligated in frame into the plasmid pQE 32 vector cut with BamHI and blunt-ended by Klenow treatment. Plasmid DNA was electroporated into competent E. coli strain XL1-Blue containing the repressor plasmid pREP4 (kanamycinR), which tightly regulates the T7 promoter in pQE 32. Transformed E. coli were plated on LB agar containing 50 μg/ml ampicillin and 10 μg/ml kanamycin. Growing colonies were picked, and minipreps were prepared to characterize the plasmid DNA. In-frame WI-1 inserts were identified by restriction analysis of plasmid DNA and confirmed by sequence analysis.

For expression of recombinant WI-1, transformed E. coli was grown in LB media containing 100 μg/ml ampicillin and 25 μg/ml kanamycin in a gyratory shaker at 37 °C. When the density of the culture reached A600 of 0.6 (after about 6 h of growth), 5 ml isopropyl-1-thio-β-D-galactopyranoside was added. Optimal production of the fusion protein was examined at 1-h intervals after induction. Bacteria were centrifuged at 5,000 x g for 15 min at 4 °C. A lysate was prepared by resuspending the pellet in 50 mM Tris, pH 8.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol equivalent to 1% of the original volume of the culture. The lysate was heated to 100 °C for 3 min and centrifuged at 12,000 x g for 1 min at room temperature. Lysates were examined by SDS-polyacrylamide gel as described by Laemmli (24). Gels were stained for protein with Coomasie Brilliant Blue.

Recombinant WI-1 containing 6 histidine residues at the N terminus was purified over Ni-NTA resin (23) according to the supplier’s specifications. To assess antibody recognition of the fusion protein, Western blots of gels were performed as described (14). Anti-WI-1 antisera used in these experiments were from immunized rabbits and blastomycosis patients in whom antibody responses to WI-1 had previously been demonstrated by radioimmunoassay (11).

Functional Assays of WI-1-mediated Attachment to Human Macrophages—WI-1-mediated attachment to human monocyte-derived macrophages was quantified by immunofluorescent staining and flow cytometry as described (15). Briefly, polystyrene microspheres (1 micron diameter; Polysciences Inc., Warrington, PA) were coated with HPLC-purified native WI-1 or recombinant WI-1 using the method of Schreiner and Horowitz (25). Microspheres (2 x 10^10) were washed twice in 0.05 mM carbonate-bicarbonate buffer, pH 9.6, and then were incubated in 1 ml of the same buffer containing 10 μg/ml of native or recombinant WI-1 or human serum albumin (HSA) as a control for 24 h at 37 °C. The microspheres were centrifuged and then were incubated for 30 min at 37 °C in 1 ml of buffer containing 0.5% Antibiotic-Antimycotic (100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml amphotericin B). The microspheres were washed twice in carbonate-bicarbonate buffer containing 0.5% HSA and standardized to 2 x 10^7/ml in Dulbecco's phosphate-buffered saline containing 0.5% HSA and 5% glycerol. The amount of WI-1 bound to microspheres was quantified by immunofluorescent staining and flow cytometry as described (15) using WI-1-reactive monoclonal antibodies to the 25-amino acid repeat (20).

Human monocytes were purified from buffy coats by sequential centrifugation on Ficoll-hypaque and Percoll gradients and cultured in RPMI 1640 containing 12.5% human serum and 10 μg/ml gentamicin. Macrophages were harvested after 4–7 days, washed, and suspended to 0.5–1.0 x 10^7/ml in Hanks' balanced salt solution containing 20 mM HEPEs, 0.25% bovine serum albumin, and 0.3 units/ml aprotinin. Five μl of macrophage suspension were added to each well of a Terasaki tissue culture plate, which had been previously coated with 1 mg/ml human serum albumin, and the cells were allowed to

1 The abbreviations used are: HPLC, high performance liquid chromatography; kb, kilobases; HSA, human serum albumin; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis.
adhere for 1 h at 37 °C.

For binding assays, 5 μl (10^5) of WI-1- or HSA-coated microspheres were added to macrophage monolayers and incubated at 37 °C for 30 min. Unattached microspheres were removed by washing, and the monolayers then were fixed with 2% glutaraldehyde, 1% sucrose in 0.01 M phosphate buffer, pH 7.2. The attachment of microspheres to macrophages was quantitated by counting the number of particles bound to 100 macrophages by phase-contrast microscopy on a Nikon diaphot-inverted microscope.

Peptide inhibition of the binding of microspheres was investigated by adding 5 μl of the inhibitor to the well just prior to the addition of the microspheres. The tandem repeat peptide inhibitor used in these experiments displays 4.5 copies of the repeat and was synthesized and purified in recombinant form as described previously (20). For monoclonal antibody blocking of macrophage receptors, 5 μl of antibody (50 μg/ml) were added to each well, and the plates were incubated for 30 min at 4 °C before microspheres were added. Monoclonal antibodies (184, specific for the common β-chain of CD18 receptors (26); 904, specific for the α-chain of CD11b/CD18 (27); 3C10, specific for CD14 (28); and 3G8, specific for the low affinity FcR of neutrophils, FcRII, CD16 (29)) were generously provided by Dr. Simon Newman (University of Cincinnati College of Medicine, Cincinnati, OH).

The results of binding assays are presented as the percentage of binding, which is the percentage of macrophages binding one or more particles, and the attachment index, which is the total number of microspheres bound per 100 macrophages. All experimental points were performed in duplicate, and experiments were repeated with macrophages from three to five different donors.

RESULTS

Identity of cDNA Probes—In a previous report (14), we described an incomplete, 1-kb WI-1 cDNA isolated from B. dermatitidis ATCC strain 60636. Subsequently, we screened additional cDNA libraries from ATCC strain 26199 to isolate the complete coding sequence for WI-1. cDNA synthesis reactions were primed with a mixture of oligo(dT) and a 30-base pair oligomer homologous to sequence at the 5' end of the available 1-kb cDNA. These efforts produced longer WI-1 cDNAs, although they were still incomplete at the 5' end. The work below was accomplished with the published 1-kb cDNA, designated BK1, and an additional larger WI-1 cDNA isolated from strain 26199, designated p1.1.2 cDNA p1.1, which overlaps with 477 nucleotides at the 5' end of cDNA BK1, extends further than BK1 toward the 5' end of WI-1 coding sequence. The overlap of these two cDNAs with respect to each other is shown in Fig. 1B. Sequence analysis of cDNA p1.1 (strain 26199) confirmed its identity as WI-1 but also demonstrated 13% sequence variation from cDNA BK1 (strain 60636) in the overlapping coding regions, indicating strain variation.2 Because cDNA p1.1 contained WI-1 coding sequence missing from cDNA BK1, we used cDNA p1.1 to further analyze genomic WI-1 by Southern analysis. cDNA p1.1 was also used to screen a genomic library made from DNA of strain 26199 to eliminate problems associated with sequence variation.

Restriction Analysis and Cloning of Genomic WI-1—We first used cDNA p1.1 as a probe in Southern analysis of 26199 genomic DNA to identify restriction fragments that might contain the complete WI-1 gene. cDNA p1.1 hybridized to a single 9.3-kb fragment of XbaI-cut genomic DNA. No smaller hybridizing fragments were observed, suggesting the gene might be intact on the 9.3-kb fragment. cDNA p1.1 also hybridized to a predicted 2.7-kb fragment of BglI-cut DNA (Fig. 1A).

Nearly 20,000 recombinants from an XbaI genomic DNA library of B. dermatitidis strain 26199 were screened by colony hybridization using p1.1 as the cDNA probe. Three independent clones were identified, and each contained the predicted 9.3-kb insert anticipated from the Southern analysis. Comparison of the three clones to WI-1 cDNAs p1.1 and BK1 by further restriction analysis and Southern hybridization confirmed the identity of the three clones as WI-1 genomic DNA.

One clone was studied more thoroughly to investigate whether the 9.3-kb genomic insert contained all of the WI-1 gene. A restriction map of the insert relative to the cDNAs is illustrated below (Fig. 1B). Overlapping WI-1 cDNAs (p1.1 and BK1) and vector sequence were used to probe Southern of a BglI digest of the clone to define the orientation of the restriction fragments and the location of the WI-1 gene. We predicted that the 3.2-kb BglI fragment was most 5' and contained 2.8 kb of DNA extending beyond the 5' end of p1.1, sufficient to contain the remainder of the WI-1 gene.

Nucleotide and Deduced Amino Acid Sequence of Genomic WI-1—The complete nucleotide and deduced amino acid sequence of genomic WI-1 is shown in Fig. 2. Several features are noteworthy. The start codon is underlined at positions 2599-2601. The open reading frame is 3438 nucleotides, and the coding sequence contains no introns. Remarkably, there are a total of 30 highly conserved repeats of a 24-amino acid sequence that comprises much of the WI-1 protein, except at the N terminus and C terminus.

In a previous report (14), we described a 25-amino acid repeat arrayed in tandem and present in 4.5 copies near the 5' end of the 1-kb WI-1 cDNA isolated from ATCC strain 60636. This repetitive sequence also was of interest for its homology over a stretch of 17 residues with the Yersinia adhesin invasin. Recently, we demonstrated that the tandem repeat promotes WI-1-mediated attachment to macrophage CD18 and CD14 receptors (15). The tandem repeat from genomic WI-1 of ATCC strain 26199 is 24 amino acids in contrast to the 25-amino acid repeat of the other strain. The sequence of the repeat of strain 26199 is HEKYDW(K/E/D/V)LW(N/D)K-YRQGDAEYQ. The alignment and homology of 17 residues of the WI-1 tandem repeat in these two fungal strains with invasin.

The 30 repeats are clustered in two noncontiguous areas of the protein. In the first area, four repeats are arrayed in tandem near the N terminus, corresponding to amino acid posi-

2 L. H. Hogan, S. Josvai, and B. S. Klein, unpublished data.
tions 211–307. In the second area, 26 repeats are arrayed in tandem corresponding to amino acid positions 407-1031. The amino acid sequence flanking these two regions also displays repeat-like sequence, but with varying amounts of degeneracy. For example, the four highly conserved repeats near the N terminus are flanked on both the 5' side (amino acid positions 182–210) and the 3' side (positions 307–335) by an elongated repeat of 28 amino acids. Even longer, more degenerate repeat-like sequence (positions 144–182) or, in some instances, short 9- or 10-amino acid elements of the tandem repeat (positions 335–406) flank these two elongated repeats of 28 amino acids and also flank the 3' end of the 26 repeats arrayed in tandem.

We previously described sequence homology of a cysteine-rich, nonrepetitive sequence near the C terminus of WI-1 cDNA BK1 with epidermal growth factor (EGF)-like domains (14). This sequence also is conserved in positions 1058–1093 of the deduced amino acid sequence of genomic WI-1 from ATCC strain 26199 (Figs. 2 and 3B).

The predicted molecular mass of the WI-1 protein coded by this nucleotide sequence is 146 kDa, which is in close agreement with the SDS-PAGE relative mobility of approximately 120 kDa for the native WI-1 protein. In addition, the deduced amino acid sequence indicates that the protein is rich in tryptophan (146 residues, 18.6 mol %), aspartic acid (161 residues, 12.7 mol %), lysine (126 residues, 11.0 mol %), tyrosine (95 residues, 10.6 mol %), and cysteine (90 residues, 6.4 mol %), as expected from previous published work on the amino acid composition of native WI-1 (20).

A Kyte-Doolittle (30) hydrophilicity plot of the deduced sequence suggests that the protein is predominantly hydrophilic, due mainly to the charge within the tandem repeat, but that the protein has a distinct hydrophobic sequence at the N-
Figure 3. Homology of WI-1 with other sequences in the GenBank database. Panel A shows sequence similarity between a 17-amino-acid portion of the tandem repeat of WI-1 (total repeat is 24 amino acids in strain 26199 and 25 amino acids in strain 60636) and Yersinia enterocolitica invasin. Vertical solid lines indicate identity between residues, vertical dashed lines indicate a conservative amino acid substitution. Panel B shows the similarity between a cysteine-rich sequence near the C terminus of WI-1 and the consensus sequence for epidermal growth factor-like domain (45). Conserved cysteines are marked with asterisks.

Figure 4. Plots of hydrophilicity and surface probability for the deduced amino acid sequence of WI-1. DNAstar was used to access the hydrophilicity plot of Kyte-Doolittle (30) and the surface probability plot of Emini (32).

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This is especially true for primary fungal pathogens, the pathogenesis of fungal infections, especially adherence mechanisms. This is especially true for primary fungal pathogens, i.e., dimorphic fungi Histoplasma capsulatum, Coccidioides immitis, and B. dermatitidis, which are the most common. These pathogens can establish themselves and invade tissues of normal hosts and often cause large and widespread outbreaks of human infection, severe manifestations of disease, or both (35–37). A better understanding of pathogenic mechanisms including adherence and adhesins in these fungi will ultimately improve treatment and control strategies. Adhesins could serve as vaccine candidates, similar to the situation in bacteria (38).

We previously showed that the 120-kDa surface protein WI-1 on B. dermatitidis mediates attachment of the yeast to human macrophages (15). In that study, WI-1-mediated binding to cells was governed, in part, by the 25-amino acid invasin-like, tandem repeat. Of interest is that the host’s humoral immune response is directed against the tandem repeat. Anti-WI-1 antibodies in human serum (14), and a panel of murine monoclonal antibodies (20) react almost exclusively with the repeat. Although cellular immunity is believed to play the key role in resistance to dimorphic fungi and other fungal pathogens, antibodies directed against the WI-1 tandem repeat may afford the host against B. dermatitidis; we speculate that interfering with attachment to host tissues may neutralize the pathogenicity of the fungus. Monoclonal antibodies directed against the capsular polysaccharide of Cryptococcus neoformans protect mice against experimental, cryptococcal infection (39), and antibodies against a mannan adhesin of C. albicans protect mice against experimental, invasive candidiasis (40). Thus, studies with B. dermatitidis, together with these on C. neoformans and C. albicans, suggest that immunity to surface components including adhesins may confer protection, and by inference, that adhesins of pathogenic fungi may be candidates for vaccine development.

In this study, we sought to clone the complete gene encoding WI-1 to characterize its binding domains at the molecular level and permit detailed analyses of WI-1-mediated adherence. Our efforts at cloning a full-length cDNA were unsuccessful but did yield a larger WI-1 cDNA that was useful for restriction analysis and screening of a genomic library to isolate full-length WI. In retrospect, a full-length cDNA was probably difficult to isolate because of the repetitive WI-1 sequence and inability to reverse transcribe it.

We isolated the full-length gene on a 9.3-kb fragment of XbaI-out genomic DNA. Several lines of evidence indicate we have cloned WI-1 rather than some other WI-1-like gene sequence. First, positive clones were isolated by colony hybridization using a WI-cDNA probe, and the sequence of genomic WI-1 to characterize its binding domains at the molecular level and permit detailed analyses of WI-1-mediated adherence. Our efforts at cloning a full-length cDNA were unsuccessful but did yield a larger WI-1 cDNA that was useful for restriction analysis and screening of a genomic library to isolate full-length WI. In retrospect, a full-length cDNA was probably difficult to isolate because of the repetitive WI-1 sequence and inability to reverse transcribe it.

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WI-1 matches that of the probe. Second, rabbit anti-WI-1 antiserum reacts strongly with recombinant WI-1 expressed as a histidine-tagged fusion protein. Finally, and most importantly, amino acid sequence from three internal peptide fragments of native WI-1 match the sequence of the genomic gene, confirming its identity as WI-1.

Several features of the WI-1 coding and upstream sequence deserve comment. To our knowledge, WI-1 is the first adhesin cloned from a pathogenic fungus and one of the few genomic genes cloned from a pathogenic yeast. This offers insight into the structure and organization of the coding and upstream sequence of genomic genes in these eukaryotic pathogens. Small introns have frequently been observed in filamentous fungi and nonpathogenic yeasts (33); none were observed in the coding sequence of WI-1.

### Table I

| Latex beads | Percentage of binding | Attachment index |
|------------|-----------------------|------------------|
| HSA-coated | 25 ± 4                | 75 ± 5           |
| nWI-1-coated | 73 ± 3               | 597 ± 81         |
| rWI-1-coated | 80 ± 4               | 629 ± 76         |

*a* nWI-1, native WI-1; *rWI-1*, recombinant WI-1.

*b* Percentages of binding and attachment index were determined as described under “Materials and Methods.”

*c* Results are mean ± S.E. (*n* = 5).

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In a study of 211 higher eukaryotic genes (41), 205 had a purine three base pairs upstream from the ATG codon. In 79% of the instances, it was an A, and this is the case for WI-1. Elements of a fungal promoter include a CAAT box, TATA box, and a CT motif; all are present in the upstream sequence of WI-1. Moreover, the spatial relationships between these elements are preserved in WI-1. In many filamentous fungal genes, the transcription start site appears in or immediately downstream from a pyrimidine-rich sequence (33). This has also been seen in yeasts, especially when genes are highly expressed or lack the usual TATA and CAAT motifs. An extreme example of a CT motif is the oliC gene of Aspergillus.
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