Antibodies to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins react with *Candida albicans* alcohol dehydrogenase

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It has been hypothesized that *Candida albicans* possesses integrin-like receptors on its cell surface. This is because *C. albicans* binds numerous fluid-phase extracellular matrix (ECM) proteins on its cell surface and adheres to the same ECM proteins when immobilized. In addition, numerous antibodies to human integrins (receptors for ECM proteins) bind to the fungal cell surface and in so doing inhibit the binding of the respective proteins. To demonstrate the presence of such a cell surface integrin, a cDNA library of *C. albicans* yeast cells was screened with polyclonal antiserum to the human fibronectin receptor ($\alpha_5\beta_1$ integrin). Clones isolated by this screening technique also reacted specifically to antiserum against the human vitronectin receptor ($\alpha_v\beta_3$ integrin). DNA sequence analysis of the cloned insert predicted a 350 aa protein (37 kDa). This predicted protein showed 75% homology at the nucleotide sequence level to alcohol dehydrogenase (ADH) of *Saccharomyces cerevisiae*. *In vitro* transcription/translation of the cloned inserts yielded a 37 kDa protein that was immunoprecipitated with antibodies to the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins and an antibody to a *C. albicans* fibronectin receptor. These antibodies and an mAb to the human vitronectin receptor demonstrated an antigen of ~37 kDa present in the cell-wall preparations of *C. albicans* and in spent growth medium. All four antibodies reacted with authentic ADH. The possible significance of these results in relation to *C. albicans* adherence is discussed.

Keywords: *Candida albicans*, adherence, integrins, extracellular matrix proteins, alcohol dehydrogenase

INTRODUCTION

Adherence of *Candida albicans* to host tissue is thought to be an important event in the pathogenesis of all forms of candidiasis and one of the proposed targets of adherence is the extracellular matrix (ECM) (Calderone & Braun, 1991; Klotz, 1992). Several groups of investigators have reported *C. albicans* receptors and/or adhesins for a number of plasma and ECM proteins. ECM protein receptors described on the surface of *C. albicans* include vitronectin (Jakab *et al.*, 1993), laminin (Bouchara *et al.*, 1990), entactin (Lopez-Ribot & Chaffin, 1994), collagen (Klotz, 1994), fibronectin (Klotz & Smith, 1991; Negre *et al.*, 1994) and tenascin-C (Lopez-Ribot *et al.*, 1999).

Receptors that bind these proteins are termed integrins,

cell-surface heterodimeric membrane glycoproteins involved in cell–cell and cell–substratum attachment (Ruoslahti, 1991). Interestingly, a number of polyclonal and monoclonal antibodies (pAbs, mAbs) to human integrins bind in a specific manner to the surface of *C. albicans*. For example, an antibody to the human fibronectin receptor, $\alpha_5\beta_1$, binds to the surface of *C. albicans* as determined by FACS analysis (Santoni *et al.*, 1994) and in so doing inhibits the binding of fibronectin to the surface of *C. albicans* yeast cells (Klotz & Smith, 1991) and the adherence of yeast cells to immobilized fibronectin (Santoni *et al.*, 1994) and to human endothelial cells (Frey *et al.*, 1990). This same antibody, when labelled with fluorescein, is distributed in patches over the surface of the fungus (unpublished data). Similar results have been obtained using antibodies to the human vitronectin receptor (Spreghini *et al.*, 1999).

Because *Candida* binds ECM proteins and antibodies to human integrins bind to *C. albicans*, a reasonable

Abbreviations: ADH, alcohol dehydrogenase; ECM, extracellular matrix; pAb/mAb, polyclonal/monoclonal antibody.

speculation is that integrin-like molecules are present on the surface of this fungus (Gustafson *et al.*, 1991). We have screened a *C. albicans* cDNA library using polyclonal antiserum to the human fibronectin receptor and isolated cDNA clones which encode an alcohol dehydrogenase (ADH) homologous to ADH of *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. We describe the cloning and characterization of this gene in addition to the possible adherence function of this extracellular protein.

METHODS

Strains and media. *Escherichia coli* strains XL-1 Blue, SOLR, TOPP-2 and SURE were all obtained from Stratagene, stored in buffered glycerol at -20°C and grown in Luria broth (LB) or on LB solidified with 1.5% (w/v) agar (Difco) at 37°C . *C. albicans* was cultured in Sabouraud dextrose (SD) broth (Difco) and maintained on SD solidified with 1.5% (w/v) agar.

cDNA libraries. A yeast cell library of *C. albicans* strain B311A (titre 1.6×10^{11} p.f.u. ml^{-1}) was a gift of G. Livi, Beecham SmithKline, Philadelphia, PA, USA. A germ tube library (titre 4×10^{10} p.f.u. ml^{-1}) was a gift of W. Fonzi, Georgetown University, Washington, DC, USA. Both libraries were prepared in λ ZAP II (Stratagene) and propagated in *E. coli* strain XL-1 Blue.

cDNA library screening. Screening of both cDNA libraries was carried out by plating on NZYM medium with duplicate nitrocellulose filters and represented approximately 10000 recombinants (Sambrook *et al.*, 1989). Briefly, phage lysates diluted to 10^{-4} p.f.u. ml^{-1} were used to infect *E. coli* XL-1 Blue and were plated on NZY plates and allowed to grow at 37°C for 3–4 h before being overlaid with IPTG-soaked (5 mM) filters and incubated for an additional 4 h at 37°C . Non-specific binding sites were blocked with Superblock (SB) in TBS (Pierce) for 30 min at ambient temperature. Rabbit polyclonal antiserum to the human fibronectin or vitronectin receptors (1:500 dilution in SB) was allowed to react with the filters at 4°C for 18 h. Filters were then washed with TBS and developed using an anti-rabbit alkaline-phosphatase-conjugated second antibody (Pierce) with BCIP/NBTZ as substrates.

Isolation of cDNA clones. pBluescript plasmids were excised from positive phage clones according to manufacturer's (Stratagene) instructions. Briefly, λ ZAP II clones (titre $\sim 10^6$ p.f.u. ml^{-1}) were combined with ExAssist helper phage and *E. coli* XL-1 Blue cells and allowed to co-infect. The resulting lysate was used to infect *E. coli* strain SOLR and cells harbouring the plasmid were selected on LB/ampicillin plates ($100 \mu\text{g ml}^{-1}$). Plasmids were purified and inserts characterized by restriction enzyme analysis in agarose gels. DNA stocks were stored frozen in $1 \times \text{TE}$ buffer (pH 8.0) or precipitated in ethanol at -20°C .

Southern blots with cDNA probes. DNA was transferred to nitrocellulose membranes (Transblot; Bio-Rad) according to the method of Southern (1975). Complementary DNA probes to human integrin subunits α_5 and β_1 (Gibco-BRL) were used against Southern blots of pBluescript library clones. Probes were labelled by the random priming method (Gibco-BRL) and labelled to a specific activity of greater than 10^7 d.p.m. ($\mu\text{g DNA}$) $^{-1}$. Hybridization was carried out in $6 \times \text{SSC}$, $5 \times \text{Denhardt's}$, 0.5% SDS buffer in 50% formamide at 42°C

in a rotisserie oven. Blots were washed under relaxed conditions in $2 \times \text{SSC}$ and 2% SDS at room temperature, dried and exposed to Kodak X-Omat film at -70°C .

In vitro translation. Approximately 1 μg supercoiled DNA from each of the plasmid clones was utilized in an *in vitro* coupled transcription/translation system using an *E. coli* S-30 extract from Promega and ^{35}S -methionine (Amersham; $> 1000 \text{ Ci mmol}^{-1}$). A sample containing 50000 TCA-precipitable d.p.m. was used in each lane of an SDS-polyacrylamide gel. Gels were developed with En 3 Hance according to manufacturer's directions (Dupont NEN), then dried at 60°C and exposed to X-ray film at -70°C .

Antibodies and reagents. The antibody to the *C. albicans* fibronectin receptor was obtained in the following manner. *C. albicans* cell-wall protein eluted with EDTA from a fibronectin affinity column was HPLC-purified (Klotz *et al.*, 1994) and used as antigen in complete Freund's adjuvant to generate polyclonal antiserum to the adhesin in New Zealand white rabbits (Research Genetics). Anti-human vitronectin receptor, polyclonal and monoclonal (clone P1F6) antisera, as well as anti-human fibronectin receptor polyclonal antiserum were purchased from Gibco-BRL. Alkaline-phosphatase-coupled secondary antibodies were obtained from Pierce (goat anti-rabbit, rabbit anti-mouse). Immunoprecipitation of the product of transcription/translation was accomplished by attaching the respective antibodies to magnetic beads (as per the instructions of the manufacturer, Dynal). Antigen was eluted from the beads and protein electrophoresis was performed.

The monospecific antibody to ADH was prepared in the following manner. Approximately 100 μg *S. cerevisiae* ADH was run on an SDS slab gel, transferred to nitrocellulose and blocked with 3% bovine serum albumin. Antiserum to the *C. albicans* fibronectin adhesin was diluted 1:500 with PBS and incubated with the antigen for 5 h at 26°C . The nitrocellulose strip was washed and stained with alkaline-phosphatase-labelled anti-rabbit antibody. A strip corresponding to the detected antigen was cut out and the antibody was eluted with 0.2 M glycine, 1 mM EGTA (pH 2.8), transferred to a microfuge tube and brought to neutrality with Tris base (Harlow & Lane, 1988).

ADH type I from *S. cerevisiae* was purchased from Boehringer Mannheim. Cell-wall extracts of *C. albicans* were prepared by octylglucopyranoside extraction as reported previously (Klotz *et al.*, 1993).

Sequence analysis. Dideoxy sequence analysis was performed according to the method of Sanger *et al.* (1977) using T7 DNA polymerase (Sequenase; USB).

RESULTS

The yeast cell and germ tube cDNA libraries were screened with polyclonal antiserum to the human fibronectin receptor $\alpha_5\beta_1$. Both libraries expressed clones reactive to the antibody. Twelve clones were isolated from the yeast cell library that were specifically recognized by the antibody. The clones identified by the pAb to the human fibronectin receptor ($\alpha_5\beta_1$) were even more strongly reactive to pAb to the human vitronectin receptor ($\alpha_v\beta_3$). We then probed these isolates for identity with the human fibronectin receptor. Southern hybridization performed with oligonucleotides of the β_1

Fig. 1. Immunoblot demonstrating the presence of a protein consistent with ADH in the cell-wall preparations of *C. albicans* using anti-integrin antibodies. Lanes: 1, *S. cerevisiae* ADH1; 2, detergent-extracted cell wall of *C. albicans* (12% SDS-PAGE reduced with DTT). The arrow points to 37 kDa. The preparations were immunoblotted with (a) pAb to the human fibronectin receptor $\alpha_5\beta_1$, (b) pAb to the human vitronectin receptor $\alpha_v\beta_3$, (c) pAb to the 60 kDa fibronectin adhesin of *C. albicans* and (d) mAb to the human vitronectin receptor (P1F6).

and α_5 chains of the human fibronectin receptor under conditions of low stringency, however, did not detect homology between the isolated clones and the human integrins. Furthermore, the same 12 clones did not react with Mo-1 or OK-M1 mAbs which recognize the human iC3b receptor [an integrin ($\alpha_M\beta_2$)]. Mo-1 and OK-M1 bind in a specific manner to the surface of *C. albicans* and in so doing inhibit the binding of complement to the fungal cell surface (Gustafson *et al.*, 1991). These results indicate that the gene and its encoded protein recognized by the antibody to the human fibronectin receptor do not share homology with the human fibronectin receptor gene, nor do all anti-integrin antibodies recognize the protein, since Mo-1 and OK-M1 do not bind.

Plasmids from the 12 clones which bound the antibodies to human fibronectin and vitronectin receptors were excised from λ ZAP II bacteriophage vectors for further characterization. The nucleotide sequence of the first 200–300 bp of each was determined and the clones were placed into four groups based on the similarity of these sequences. Expression of the proteins from these clones was accomplished using an *in vitro* coupled transcription/translation system. Clones from only two of the groups yielded protein products in the *in vitro* translation system. The expressed protein products were 37 kDa. This protein was immunoprecipitated using polyclonal sera to human fibronectin and vitronectin receptors and the *C. albicans* fibronectin adhesin (data not shown). Thus these three antibodies recognize not only the protein expressed by *Escherichia coli*, but also the protein expressed *in vitro*.

Nucleotide sequence analysis of one of the clones revealed a 37 kDa protein with > 70% homology with ADH from *S. cerevisiae* (ADH1) and *K. lactis* (ADH1, ADH3), and 61% identity with the *Schizosaccharomyces pombe* ADH gene (the sequence is assigned NCBI accession no. U15924; the online version of this paper at <http://mic.sgmjournals.org> contains a supplementary figure showing the sequence alignment). The sequence possessed no pre-sequences identified for organelle targeting or membrane insertion, e.g. the mitochondrial presequence of the *K. lactis* ADH3 gene. The *C. albicans* gene encodes a 350 aa polypeptide with a calculated

mass of 36866 Da. The sequence displays the consensus pattern for zinc-containing ADHs with the histidine in the second position of the signature predicted to be the zinc-coordinating ligand. The sequence contains 13 potential myristic acid sites and two sites for N-glycosylation. A portion of this sequence has been reported by Shen *et al.* (1991) from nt 310 to 940. Interestingly, the latter authors obtained this partial sequence after probing a *C. albicans* cDNA library with mAbs to a major allergen of *C. albicans*. Their antisera would presumably be recognizing a cell-surface-associated or extracellular protein of the fungus.

We then turned our attention to demonstrating whether a protein of the size of the encoded protein could be demonstrated in the cell wall of *C. albicans*. ADH of *S. cerevisiae* and cell-wall extracts of *C. albicans* were electrophoresed and analysed by Western blotting with pAbs to the human fibronectin receptor, the human vitronectin receptor and the *C. albicans* fibronectin adhesin, and with an mAb to the human vitronectin receptor (Fig. 1). These same antibodies had immunoprecipitated the *in vitro* transcription/translation product of the gene and/or identified the clones in the original screening. Each antibody reacted with bona fide *S. cerevisiae* ADH (arrow). These antibodies also reacted to multiple proteins from detergent extracts of *C. albicans*. However, each antibody demonstrated the presence of a cell-wall antigen of about 37 kDa, similar to ADH. The secondary antibodies did not react with the enzyme, nor with the *C. albicans* cell-wall proteins (data not shown). Furthermore, a pAb to the *C. albicans* fibronectin adhesin, which immunoprecipitates the encoded protein and binds to bona fide ADH, also binds to the surface of yeast cells (Fig. 2). Thus, the antibody data demonstrate that an antigen consistent with the size of ADH is found on the cell surface of *C. albicans*.

Next we asked the question whether the antigen is secreted extracellularly. To answer this we used a monospecific antibody to ADH that was derived from the anti-fibronectin adhesin antibody. Supernatant from the growth medium of *C. albicans* was immunoblotted and reacted with the monospecific antibody to ADH.

Fig. 2. Photomicrograph of *C. albicans* yeast cells stained with antibody prepared to protein bound by fibronectin. (a) Yeast cells stained with fluorescein-tagged goat anti-rabbit antibody; (b) the same cells reacted with a pAb to the anti-fibronectin receptor and a fluorescein-tagged second antibody; (c) different field of cells prepared in the same manner as (b).

Fig. 3. Immunoblot of *C. albicans* growth medium. (a) Authentic ADH (37 kDa; arrow); (b) supernatant of minimal broth medium in which *C. albicans* had grown for 24 h. These were immunoblotted with a pAb to the *C. albicans* fibronectin adhesin.

Fig. 3 demonstrates the presence of a 37 kDa protein in the supernatant, a size and antibody reaction compatible with the protein being ADH.

DISCUSSION

Plasma fibronectin was the first ECM protein reported to bind *C. albicans* (Skerl *et al.*, 1984) and an expanding number of publications have followed describing ECM

protein interactions with the fungus (Jakab *et al.*, 1993; Klotz, 1994; Klotz & Smith, 1991; Lopez-Ribot *et al.*, 1999, 1994; Lopez-Ribot & Chaffin, 1994; Negre *et al.*, 1994; Santoni *et al.*, 1994; Spreghini *et al.*, 1999). A pAb to the human integrin, $\alpha_5\beta_1$ (a fibronectin receptor), inhibits the binding of fluid-phase fibronectin to the surface of *C. albicans* (Klotz & Smith, 1991), inhibits the adherence of the yeast cell to human endothelial cells (Frey *et al.*, 1990) and to immobilized fibronectin (Santoni *et al.*, 1994), and does so by binding to the surface of the fungus. This same antibody was used in this work to identify the cDNA clones which expressed ADH. In addition to the integrin antibodies used in this work and that of Santoni *et al.* (1994) and Spreghini *et al.* (1999), cDNA libraries of *C. albicans* have been probed successfully with an mAb to $\alpha_5\beta_1$ (Santoni *et al.*, 1993) and with an antibody to the transmembrane portion of the β_1 protein of the $\alpha_5\beta_1$ integrin (Marcantonio & Hynes, 1988). Recently, an integrin-like gene, *INT1*, has been described in *C. albicans* by screening *C. albicans* genomic DNA with a cDNA probe from the transmembrane domain of the human integrin, α_M (Gale *et al.*, 1996). The homologue of this protein is confined to this small domain. The N terminus does not possess homology with an integrin.

Because of our prior work with the fibronectin receptor of *C. albicans*, we attempted the detection of a fibronectin-binding integrin in *C. albicans*. Multiple clones from a *C. albicans* yeast cell cDNA library were isolated using polyclonal serum to the human fibronectin receptor. The clones also reacted strongly to polyclonal serum to the human vitronectin receptor. These clones were probed by Southern hybridization with the human α_5 and β_1 chains of the human fibronectin receptor ($\alpha_5\beta_1$) and no homology was detected, suggesting that although similarity exists at the level of protein structure there is no homology at the DNA sequence level. The protein encoded by the clones is an ADH with a homology of $\sim 75\%$ with ADH1 of *S. cerevisiae* and ADH3 of *K. lactis*. pAbs to the human fibronectin receptor, vitro-

nectin receptor and the *C. albicans* fibronectin adhesin, and an mAb to the human vitronectin receptor reacted strongly to pure ADH and the protein encoded by the isolated clones. Furthermore, a monospecific antibody demonstrated that *C. albicans* ADH appears in the growth medium.

The gene reported here does not contain a secretory leader sequence; therefore the manner in which it is secreted to the cell surface is unknown. However, *C. albicans* 3-phosphoglycerate kinase appears on the surface of *C. albicans* yet lacks a secretory leader sequence (Alloush *et al.*, 1997). GAPDH of *Schistosoma mansoni* (Goudot-Crozel *et al.*, 1989) and ADH of *Entamoeba histolytica*, which is a putative ECM adhesin (Yang *et al.*, 1994), are found on the cell surface of these micro-organisms but also lack a leader sequence. In the absence of a signal sequence it is unknown how these particular proteins get to the surface of micro-organisms. However, multiple means exist for the secretion of peptides lacking a leader sequence, such as the use of 'traffic wardens' (Salmond & Reeves, 1993), charged amino acids in the sequence (Salmond & Reeves, 1993) or even the presence of certain amino acids in the C terminus of the protein may determine extracellular secretion (Stanley *et al.*, 1991).

The possibility that a cell-surface-associated cytosolic enzyme could serve in the capacity of an ECM protein receptor and/or adhesin is well accepted in other micro-organisms. For example, *E. histolytica* ADH binds fibronectin, collagen type II and laminin (Yang *et al.*, 1994). Along similar lines, the fibronectin receptor of *Streptococcus pyogenes* has been shown to be a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Pancholi & Fischetti, 1992). Furthermore, these cytosolic enzymes are found on the cell surface. GAPDH is a major protective antigen found on the surface of *Schistosoma mansoni* (Goudot-Crozel *et al.*, 1989). GAPDH also functions as a surface lectin responsible for flocculation of the yeast *Kluyveromyces marxianus* (Fernandes *et al.*, 1992). Pertinent to *C. albicans* it has been shown that the immunodominant *C. albicans* glycolytic enzyme enolase is found in culture supernatants and on the surface of the fungus (Sundstrom & Aliaga, 1994), and the cytosolic enzyme 3-phosphoglycerate kinase is located on the surface of the fungus (Alloush *et al.*, 1997). GAPDH of *C. albicans* is cell-wall-associated and even binds fibronectin and laminin (Gozalbo *et al.*, 1998). These examples of cytosolic enzymes performing in different capacities are perhaps examples of gene sharing, a concept best demonstrated in the vertebrate cornea and lens where the enzyme ADH serves as a major structural protein with little or no enzymic activity (Cooper *et al.*, 1993).

Our preliminary data in this report suggest that *C. albicans* ADH is found on the cell surface and in the culture supernatant. This protein was identified by screening a *C. albicans* cDNA library with antibodies to human integrins which are known to bind to the fungal cell surface. The cross-reactivity of human integrin

antibodies with this fungal protein is quite likely to be the explanation for the phenomenon of integrin antibodies binding to the *C. albicans* cell surface. This will need to be confirmed by examining antibody reactivity in cells deleted for both copies of the ADH gene. Although ADH added exogenously did not inhibit adherence of *C. albicans* to fibronectin, this fact does not rule out ADH as an adhesin. There are other *C. albicans* adhesins whose function is not inhibited upon the addition of excess ligand (e.g. Gozalbo *et al.*, 1998 or Gaur & Klotz, 1997). Therefore, whether or not ADH serves as a receptor for fibronectin or vitronectin remains unknown.

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