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Infect. Immun. **2006, 74(1):382. DOI: 10.1128/IAI.74.1.382-389.2006.**

INFECTION AND IMMUNITY, Jan. 2006, p. 382–389 Vol. 74, No. 1
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.1.382–389.2006

Glyceraldehyde-3-Phosphate Dehydrogenase of *Paracoccidioides brasiliensis* Is a Cell Surface Protein Involved in Fungal Adhesion to Extracellular Matrix Proteins and Interaction with Cells

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Received 1 September 2005/Returned for modification 14 September 2005/Accepted 24 October 2005

The pathogenic fungus *Paracoccidioides brasiliensis* causes paracoccidioidomycosis, a pulmonary mycosis acquired by inhalation of fungal airborne propagules, which may disseminate to several organs and tissues, leading to a severe form of the disease. Adhesion to and invasion of host cells are essential steps involved in the infection and dissemination of pathogens. Furthermore, pathogens use their surface molecules to bind to host extracellular matrix components to establish infection. Here, we report the characterization of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *P. brasiliensis* as an adhesin, which can be related to fungus adhesion and invasion. The *P. brasiliensis* GAPDH was overexpressed in *Escherichia coli*, and polyclonal antibody against this protein was obtained. By immunoelectron microscopy and Western blot analysis, GAPDH was detected in the cytoplasm and the cell wall of the yeast phase of *P. brasiliensis*. The recombinant GAPDH was found to bind to fibronectin, laminin, and type I collagen in ligand far-Western blot assays. Of special note, the treatment of *P. brasiliensis* yeast cells with anti-GAPDH polyclonal antibody and the incubation of pneumocytes with the recombinant protein promoted inhibition of adherence and internalization of *P. brasiliensis* to those in vitro-cultured cells. These observations indicate that the cell wall-associated form of the GAPDH in *P. brasiliensis* could be involved in mediating binding of fungal cells to fibronectin, type I collagen, and laminin, thus contributing to the adhesion of the microorganism to host tissues and to the dissemination of infection.

Paracoccidioides brasiliensis is a dimorphic fungal pathogen, the etiological agent of paracoccidioidomycosis (PCM), endemic in Latin America. The disease begins in the lungs and then disseminates to other organs and systems (14). The pathogen apparently has its natural habitat in soil, and mainly rural workers appear to become infected by inhalation of fungal airborne microconidia, which reach the pulmonary alveolar epithelium and transform into the parasitic yeast form. Over 10 million people in areas where the pathogen is endemic could be infected with *P. brasiliensis*, 2% of which are presumed to develop PCM (25).

The development of PCM depends on interactions between fungal and host components. *P. brasiliensis* can parasitize various tissues. The ability of pathogens to bind to components of the extracellular matrix (ECM) has been described as an important mechanism in the invasion of host tissues (18, 21). Just as with many pathogenic microorganisms, adhesion of *P. brasiliensis* to the host surface is thought to be a crucial step in the pathogenic process and a prerequisite for host colonization. *P. brasiliensis* expresses proteins that interact in various ways with the extracellular environment. For instance, the glycoprotein gp43 has been described as a mediator molecule in the binding of *P. brasiliensis* to laminin (37). A 30-kDa protein which is highly expressed in *P. brasiliensis* isolated from animals, presenting a high capacity to adhere to and invade Vero cells, has properties of an adhesin (2). A protein of 32 kDa present in the cell wall and characterized as a hypothetical molecule from *P. brasiliensis* was recently described as able to interact with the ECM components (17). Despite those few descriptions, the regulation and mechanistic details of *P. brasiliensis* in vitro and in vivo cellular adhesion and infection remain poorly understood.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) constitutes a protein family which displays diverse activities in different subcellular locations, in addition to its well-characterized role in glycolysis (35). Specific intracellular interactions seem to be related to the new activities of GAPDH. Accordingly, membrane-bound GAPDH of group A streptococci has been reported to bind fibronectin, lysozyme, and the cytoskeletal proteins myosin and actin, indicating that it may function in the colonization of those bacteria (30). Also, GAPDH of *Streptococcus oralis* was described as a dominant receptor for *Porphyromonas gingivalis* fimbriae, contributing to host colonization by the latter microorganism (24).

Cell wall-associated GAPDHs have been described for

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fungi. With *Candida albicans*, it has been demonstrated that an immunogenic and enzymatically active GAPDH is found at the cell surface (16) and that clinical strains express this surface antigen both in vitro and in infected tissues (15). In addition, the cell wall-associated GAPDH of *C. albicans* is a fibronectin and laminin binding protein (19). Proteomic and biochemical analysis indicated that GAPDH is also a cell surface plasminogen binding protein of *C. albicans*, which could potentially increase the fungus capacity for tissue invasion and necrosis (8).

We have previously identified by immunoproteomic sequencing and microsequencing of peptides two isoforms of 36 kDa, with pIs of 6.8 and 7.0, of the *P. brasiliensis* GAPDH (3, 13). We have also characterized the cDNA and genomic clones encoding the homologue of GAPDH. We have previously provided insights into the structure, function, and potential regulation of *GAPDH*, a single-copy gene of *P. brasiliensis* which is conserved across species. We have also demonstrated that the expression of GAPDH and its transcript are more abundant in the parasitic yeast phase of *P. brasiliensis* (3). According to these data, this protein seems to play a role in the fungus parasitic yeast phase and thus should be better characterized.

In the present study, we report the heterologous overexpression of *P. brasiliensis* recombinant GAPDH and its purification. In addition, we demonstrate for the first time the presence of this protein in the glycolytic pathway in the cell wall of *P. brasiliensis*. We have also demonstrated that cell wall-associated GAPDH is able to bind to extracellular matrix components. The protein seems to mediate the processes of adherence and internalization of *P. brasiliensis* to in vitro-cultured cells, as suggested by the abilities of the anti-GAPDH polyclonal antibody and the recombinant protein to interfere with both processes. These data suggest that GAPDH may play a role in mediating the attachment and internalization of the fungus to host tissues, potentially playing a role in the establishment of disease.

MATERIALS AND METHODS

***P. brasiliensis* isolate and growth conditions.** The *P. brasiliensis* Pb01 isolate (ATCC MYA-826) has been previously investigated by our laboratory and was cultivated in semisolid Fava-Neto's medium (12) at 36°C for the yeast form and at 22°C for the mycelium phase.

Cloning cDNA containing the complete coding region of GAPDH into expression vector. The cloned cDNA containing the complete coding region of GAPDH (GenBank accession number AY061958) (3) was amplified by PCR using oligonucleotide sense (5′ CACCATGGTCGTCAAGGTTGG 3′) and antisense (5′ GCTGCGAATTCCTATTTGCCAGC 3′) primers. The sequence CACC (underlined) was incorporated at the 5′ end of the sense primer. The amplification parameters were as follows: an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min and 30 s. A final elongation step was performed at 68°C for 1 min. The resulting 1,000-bp product was subcloned into the TOPO pET100 expression vector (Invitrogen, Life Technologies) to yield the TOPO-pET100-GAPDH construct. The recombinant plasmid was used to transform *Escherichia coli* XL1-Blue competent cells by the heat shock method (34). Ampicillin-resistant transformants were cultured, and plasmid DNA was analyzed by PCR.

Heterologous expression of *P. brasiliensis* GAPDH and recombinant protein purification. Bacteria transformed with the TOPO-pET-100-GAPDH construct were grown in LB medium supplemented with ampicillin (100 μg/ml) at 37°C until the optical density at 600 nm reached 0.6. Synthesis of the recombinant protein was then initiated by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.8 mM to the growing culture. After 2 h, the bacterial cells were harvested by centrifugation at 3,000 × *g* and lysis was achieved by incubation of the cells with guanidinium lysis buffer (6 M guanidine hydrochloride, 20 mM $NaPO_4$, pH 7.8, 500 mM NaCl) and sonication. The recombinant protein containing the His tag at its N-terminal end was purified on Ni-nitrilotriacetic acid resin (Invitrogen) under hybrid conditions, as follows. The cell lysate was passed through a Ni-nitrilotriacetic acid column equilibrated with denaturing binding buffer (8 M urea, 20 mM $NaPO_4$, pH 7.8, 500 mM NaCl). The column was washed sequentially with denaturing binding buffer, denaturing wash buffer (8 M urea, 20 mM $NaPO_4$, pH 6.0, 500 mM NaCl), and finally native wash buffer (50 mM $NaPO_4$, 0.5 M NaCl, 20 mM imidazole). The bound proteins were eluted with native elution buffer (40 mM $NaPO_4$, 0.4 M NaCl, 600 mM imidazole) to refold the protein, by following the manufacturer's instructions. The purity and size of the protein were evaluated by running the purified molecule through 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining.

Antibody production. The purified recombinant GAPDH was used to generate specific rabbit polyclonal serum. Rabbit preimmune serum was obtained and stored at −20°C. The purified protein (300 μg) was injected into rabbit with Freund's adjuvant three times at 2-week intervals. The obtained serum, containing monospecific anti-GAPDH polyclonal antibodies, was sampled and stored at −20°C.

Preparation of *P. brasiliensis* cell extracts. Yeast and mycelium protein crude extracts were obtained by disruption of frozen cells in the presence of protease inhibitors *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (50 μg/ml), 4-chloromercuribenzoic acid (1 mM), leupeptin (20 mM), phenylmethylsulfonyl fluoride (20 mM), and iodoacetamide (5 mM) in homogenization buffer (20 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$). The mixture was centrifuged at 12,000 × *g* at 4°C for 10 min, and the supernatant was used for further analysis of proteins by one-dimensional gel electrophoresis.

Obtaining cell extracts. Cell extracts were prepared from yeast cells, as described elsewhere (2, 5). In brief, yeast cells of *P. brasiliensis* were grown for 7 days in solid medium as described above. Cells (300 mg) were resuspended in 1.0 ml of 10 mM phosphate-buffered saline (PBS), pH 7.2, and vortexed for 30 s. The cells were centrifuged for 1 min at 560 × *g*, and the supernatant was collected and used for further analysis.

Electrophoretic analysis. Electrophoresis of native proteins and SDS-PAGE were performed according to O'Farrell (29) and Laemmli (23), respectively. The proteins were precipitated by addition of 10% (wt/vol) trichloroacetic acid, and the pellets were washed in 10% (vol/vol) cold acetone. The samples were resuspended in lysis buffer containing 9.5 M urea, 2% (vol/vol) Nonidet P-40, 5% (vol/vol) β-mercaptoethanol, and ampholines at pH ranges of 5.0 to 8.0 and 3.5 to 10.0 (ratio 4:1). The proteins were Coomassie blue or silver stained.

Western blot analysis. The protein extracts were resolved by one- or two-dimensional gel electrophoresis. The proteins were electrophoretically transferred to a nylon membrane and checked by Ponceau S to determine equal loading. GAPDH was detected with the polyclonal antibody raised to the recombinant protein. After reaction with alkaline phosphatase anti-rabbit immunoglobulin G (IgG), the reaction was developed with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP-NBT). Negative controls were obtained with rabbit preimmune serum.

Transmission electron microscopy of *P. brasiliensis* yeast cells and immunocytochemistry of the GAPDH protein. Yeast cells of *P. brasiliensis* were fixed overnight at 4°C in solution containing 2% (vol/vol) glutaraldehyde, 2% (wt/vol) paraformaldehyde, and 3% (wt/vol) sucrose in 0.1 M sodium cacodylate buffer at pH 7.2. After fixation, the yeast cells were rinsed in the same buffer and postfixed for 1 h in solution containing 1% (wt/vol) osmium tetroxide, 0.8% (wt/vol) potassium ferricyanide, and 5 mM $CaCl_2$ in sodium cacodylate buffer, pH 7.2. The material was dehydrated in a series of ascending acetones (30 to 100%) (vol/vol) and embedded in Spurr resin (Electron Microscopy Sciences, Washington, Pa.). Ultrathin sections were stained with uranyl acetate, 3% (wt/vol), and lead citrate (33). The material was observed with a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan).

For ultrastructural immunocytochemistry studies, yeast cells were fixed in a mixture containing 4% (wt/vol) paraformaldehyde, 0.5% (vol/vol) glutaraldehyde, and 0.2% (wt/vol) picric acid in 0.1 M sodium cacodylate buffer at pH 7.2 for 24 h at 4°C. The cells were rinsed several times using the same buffer, and free aldehyde groups were quenched with 50 mM ammonium chloride for 1 h, followed by block staining in solution containing 2% (wt/vol) uranyl acetate in 15% (vol/vol) acetone for 2 h at 4°C (4). The material was dehydrated in a series of ascending concentrations of acetone (30 to 100%) (vol/vol) and embedded in LR Gold resin (Electron Microscopy Sciences, Washington, Pa.).

The ultrathin sections were collected on nickel grids, preincubated in 10 mM PBS containing 1.5% (wt/vol) bovine serum albumin (BSA) and 0.05% (vol/vol) Tween 20, (PBS-BSA-T), and subsequently incubated for 1 h with the polyclonal

antibody against the recombinant GAPDH (diluted 1:100). After being washed with PBS-BSA-T, the grids were incubated for 1 h with the labeled secondary antibody (rabbit IgG, Au conjugated, 10 nm; diluted 1:20). Subsequently, the grids were washed with the buffer described above, followed by a wash with distilled water; stained with uranyl acetate, 3% (wt/vol), and lead citrate (33); and observed with a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan). Controls were incubated with rabbit preimmune serum at 1:100, followed by incubation with the labeled secondary antibody.

The gold particles were quantified in five independent preparations of yeast cells. The particles were counted in total cell distribution, as well as in the cell wall and cytoplasm. Results were expressed as the number of gold particles, represented as the means of the counts performed three times with standard deviations included.

Affinity ligand assays. Far-Western assays were carried out as previously described (20). Recombinant GAPDH was submitted to SDS-PAGE and blotted onto nitrocellulose membranes. Blotted protein was assayed for laminin, fibronectin, and type I collagen binding as follows. After being blocked overnight with 1.5% (wt/vol) BSA in 10 mM PBS, the membranes were incubated with laminin (30 μg/ml), fibronectin (30 μg/ml), or type I collagen (20 μg/ml) diluted in PBS-BSA-T for 90 min and then washed three times (for 10 min each time) in 10 mM PBS containing 0.05% (vol/vol) Tween 20 (PBS-T). The membranes were incubated for 1 h with rabbit antibodies antilaminin, antifibronectin, or anti-type I collagen in PBS-BSA-T (diluted 1:100). The blots were washed with PBS-T and incubated with peroxidase-labeled goat anti-rabbit immunoglobulin (diluted 1:1,000). The blots were washed with PBS-T, and the reactive bands were developed with hydrogen peroxide and diaminobenzidine (Sigma-Aldrich Co.) as the chromogenic reagent. As controls, the blots were incubated only with antilaminin, antifibronectin, and anti-type I collagen antibodies, in the absence of the ECM proteins (laminin, fibronectin, and type I collagen). The positive control was obtained by incubating the recombinant GAPDH with the anti-GAPDH polyclonal antibody (diluted 1:500), and the reaction was developed as described above. Additional controls were obtained with BSA fractionated by SDS-PAGE, blotted onto nitrocellulose membranes, and incubated with the ECM proteins and the respective antibodies, as described above.

Binding assays of the recombinant GAPDH to pneumocytes. Type II pneumocyte line A549 was obtained from the American Type Culture Collection (Manassas, VA). The cells were seeded overnight in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% (vol/vol) heat-inactivated fetal calf serum. The cells were washed three times with 10 mM PBS, and DMEM not containing fetal calf serum was added. Monolayers of cells were incubated with 50 μg/ml of recombinant GAPDH at 37°C for 5 h and washed with 10 mM PBS to remove unbound protein. Next, double-distilled water was added and the cells were incubated for 4 h at room temperature to obtain total lysis. The lysates were centrifuged at 1,400 × *g* for 5 min, and the supernatant was submitted to SDS-PAGE. Proteins in the gel were transferred to membranes and incubated overnight with blocking buffer (PBS-BSA-T). The secondary antibody was alkaline phosphatase coupled with anti-rabbit IgG (Sigma-Aldrich Co.). The reactions were developed with BCIP-NBT. Negative controls were obtained by analyzing the supernatant of lysed pneumocytes not preincubated with the recombinant protein GAPDH, as well as by coating the cell culture flask with 50 μg/ml of GAPDH.

Interaction of *P. brasiliensis* with in vitro-cultured pneumocytes: inhibition of adherence and infection by GAPDH and by the antibody anti-GAPDH. A549 pneumocytes were incubated for 1 h at 37°C with the recombinant GAPDH protein (25 μg/ml), diluted in 10 mM PBS. After this incubation period, the cells were washed three times in DMEM and 10^8 yeast forms of *P. brasiliensis* were added to the epithelial cells. Incubation was performed for 2 and 5 h at 37°C to allow the processes of adhesion and infection, respectively, as described previously (2, 22, 27). Additionally, 10^8 yeast cells of *P. brasiliensis* were incubated for 1 h at 37°C with the polyclonal antibody anti-GAPDH (diluted 1:100). After that, the cells were washed three times in 10 mM PBS and allowed to interact with the A549 pneumocytes. Control experiments were performed with A549 cells not preincubated with the recombinant GAPDH protein, *P. brasiliensis* yeast cells not preincubated with the anti-GAPDH antibody, and pneumocytes preincubated with BSA (25 μg/ml). The percentages of infected cells were determined by randomly counting a minimum of 300 cells on each triplicate coverslip. The adhesion and infection indices were calculated as described previously (11, 27). Results are presented as the means of counts performed three times with standard deviations included. The adhesion index was obtained by multiplying the mean number of attached yeast cells per pneumocyte by the percentage of infected cells. The infection index (adherence plus internalization) was determined by the number of total fungi interacting with the epithelial cells 5 h after addition of the yeast cells, as previously described (22, 27). All experiments were performed in triplicate.

Statistical analysis. The means and standard deviations of at least three distinct experiments were determined. Statistical analysis was performed by using analysis of variance (*F* test followed by Duncan test). *P* values of 0.05 or less were considered statistically significant.

RESULTS

Expression and purification of recombinant GAPDH and production of polyclonal antibody. The cDNA encoding the *P. brasiliensis* GAPDH was subcloned into the expression vector TOPO pET100 to obtain the recombinant fusion protein. After induction with IPTG, a 39-kDa recombinant protein was detected in bacterial lysates (Fig. 1A, lane 3). The protein was not present in crude extracts from control cells (Fig. 1A, lane 1) or in extracts from noninduced *E. coli* cells carrying the expression vector (Fig. 1A, lane 2). The six-histidine residues fused to the N terminus of the recombinant protein were used to purify the protein from bacterial lysates by nickel-chelate affinity. The recombinant protein was eluted and fractions of equal volume (1 ml) were collected and analyzed by SDS-PAGE. Fractions showed a single band of 39 kDa (Fig. 1A, lane 4).

The purified recombinant protein was used to generate rabbit polyclonal antibody. Protein extracts of yeast and mycelium and also the purified recombinant protein were subjected to SDS-PAGE analysis and stained with Coomassie blue (Fig. 1B). The same samples were blotted to nitrocellulose membranes and reacted to the polyclonal antibody (Fig. 1C). As demonstrated, a single band of 36 kDa was detected in extracts of both yeast and mycelium (Fig. 1C, lanes 1 and 2). Recombinant GAPDH presents a molecular mass (39 kDa) higher than that of the native protein, due to the additional six-histidine residues fused to its N terminus (Fig. 1C, lane 3). No cross-reactivity to the rabbit preimmune serum was evidenced with the samples (Fig. 1D, lanes 1 to 3).

Detection of GAPDH protein in *P. brasiliensis* cell extracts. The cell extracts of yeast cells were analyzed by two-dimensional gel electrophoresis (Fig. 2A). The characterized isoforms of GAPDH, with pIs of 6.8 and 7.0, are shown in this cellular fraction. The polyclonal antibody was used for immunodetection of the native GAPDH. This antibody recognized both isoforms of the native protein in the cell extracts, which correspond to the most superficial components of the cell wall (Fig. 2B).

Detection of the GAPDH protein by immunoelectron microscopy of *P. brasiliensis* yeast cells. In order to continue to define the cellular localization of the GAPDH protein in *P. brasiliensis*, we performed immunocytochemistry experiments using ultrathin sections of LR Gold-embedded yeast cells of *P. brasiliensis*. Electron microscopy of conventionally embedded cells revealed the ultrastructure of the *P. brasiliensis* yeast form (Fig. 3A). An electron-dense cell wall and the plasma membrane appear as defined structures. The contour of the nucleus is relatively smooth and appears with condensed chromatin that is homogeneously distributed. The cytoplasm appears occupied by mitochondria and well-developed vacuoles. The immunocytochemistry assays revealed gold particles detected in the cytoplasm and extending through the cell wall, indicating the double localization of GAPDH in *P. brasiliensis* (Fig. 3B and

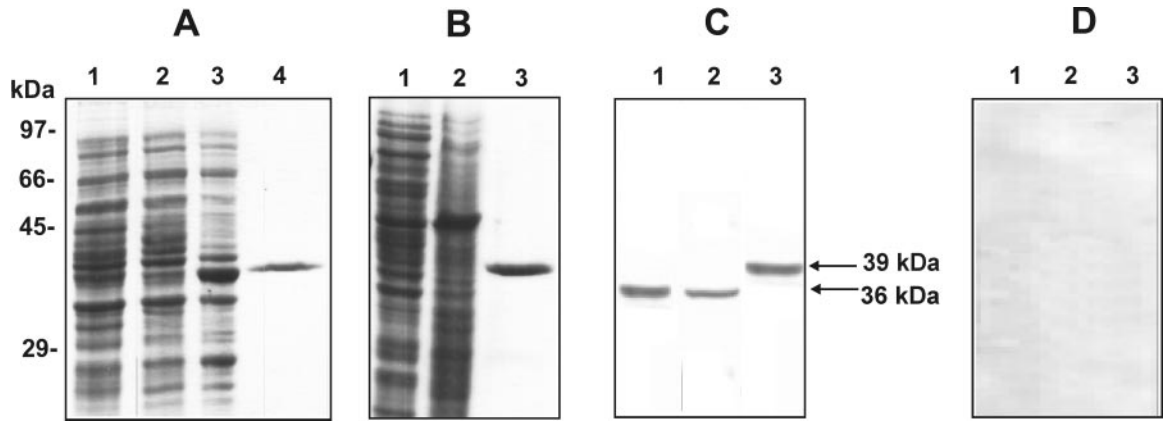

FIG. 1. Expression and purification of recombinant GAPDH and generation of rabbit polyclonal antibody. (A) SDS-PAGE analysis of *P. brasiliensis* recombinant GAPDH. *E. coli* cells harboring the TOPO-pET-100 GAPDH plasmid were grown at 37°C to an A_{600} of 0.6 and harvested before (lane 2) and after (lane 3) a 2-h incubation with 0.8 mM IPTG. The cells were lysed by extensive sonication. Lane 1, control *E. coli* cells; lane 4, affinity-isolated GAPDH. SDS–12% PAGE was carried out, and the proteins were stained by Coomassie blue R-250. (B) Electrophoretic analysis of *P. brasiliensis* proteins and recombinant GAPDH. The protein extracts were fractionated by one-dimensional gel electrophoresis and stained by Coomassie blue. Lane 1, protein extracts from yeast cells (30 μg); lane 2, protein extracts from mycelium (30 μg); lane 3, recombinant GAPDH (2.0 μg). (C and D) Western blot analysis of native and recombinant GAPDH. The same samples as for panel B were run in parallel, blotted onto a nitrocellulose membrane, and detected by using (C) rabbit polyclonal anti-recombinant GAPDH antibody or (D) rabbit preimmune serum. After reactions with the anti-rabbit IgG alkaline phosphatase-coupled antibody (diluted 1:1,000), the reactions were developed with BCIP-NBT. Molecular size markers are indicated.

C). Control samples obtained by incubation of the yeast cells with the rabbit preimmune serum were free of label (Fig. 3D).

The gold-labeled particles were counted in five immunocytochemistry assays, as shown in Fig. 4. The amount of gold particles was significantly larger in the cell wall ($P < 0.05$) than in the cytoplasmic compartment (Fig. 4).

Binding of recombinant GAPDH to extracellular matrix proteins. The ability of the recombinant GAPDH of *P. brasiliensis* to bind laminin, fibronectin, and type I collagen was determined by far-Western blotting assays, as shown in Fig. 5A. The recombinant protein presents the ability to bind to laminin (Fig. 5A, lane 1), fibronectin (lane 2), and type I collagen (lane 3). The positive control was developed with the anti-GAPDH polyclonal antibody (Fig. 5A, lane 4). Negative controls were obtained by incubating recombinant GAPDH in the absence of the ECM proteins (Fig. 5B, lanes 1 to 3), as well by incubating the recombinant molecule with just the secondary antibody (Fig. 5B, lane 4). The specificity of the binding of GAPDH to the ECM proteins was also demonstrated by the binding assay of the ECM components to BSA (Fig. 5C, lanes 1 to 3). No reactivity between BSA and the ECM proteins was demonstrated. In addition, the polyclonal antibody to GAPDH did not present cross-reactivity to BSA (Fig. 5C, lane 4).

The adhesin characteristic of GAPDH was also evaluated by interaction of the recombinant protein with pneumocytes (Fig. 5D). The purified protein behaved as an adhesin, binding to the pneumocytes (Fig. 5D, lane 1). Negative controls were developed with the pneumocytes not incubated with the recombinant GAPDH (Fig. 5D, lane 2), as well as with the recombinant protein added to the cell culture flask, in the absence of the cell monolayer (Fig. 5D, lane 3).

Inhibition of *P. brasiliensis* infection of pneumocytes, caused by recombinant GAPDH and by the polyclonal antibody anti-GAPDH. The infection index was determined by interactions

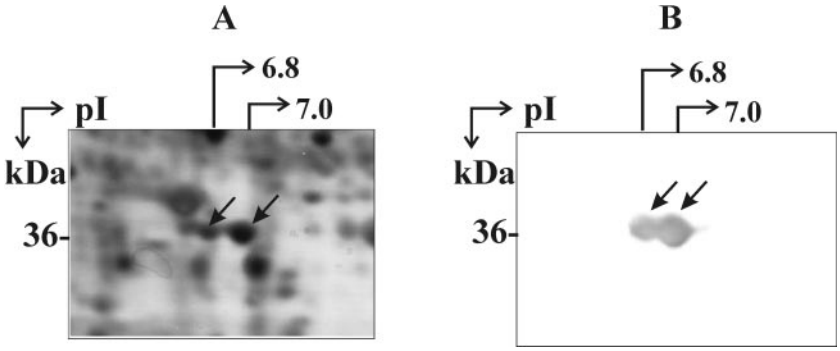

FIG. 2. Two-dimensional gel electrophoresis of *P. brasiliensis* cell extracts. Cell extracts from yeast cells of *P. brasiliensis* were obtained, and 50 μg of total proteins was loaded on two-dimensional gels. (A) SDS-PAGE gel stained with silver. (B) Reactivity of the GAPDH isoforms analyzed by Western blotting with the polyclonal antibody produced to the recombinant protein. Arrows point to the two characterized isoforms of GAPDH. Numbers to the left of both figures refer to the molecular mass of the characterized GAPDH. At the top are indicated the isoelectric points of both protein isoforms.

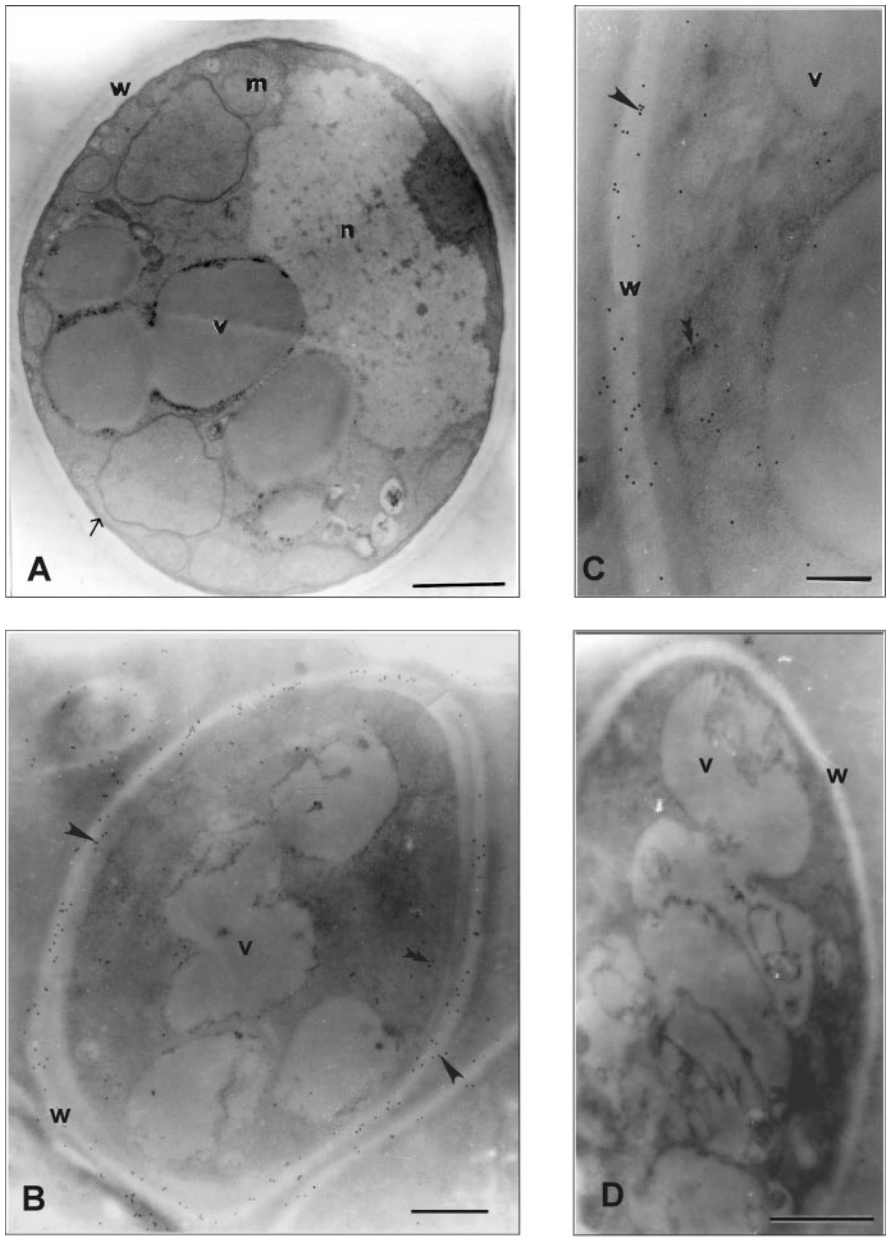

FIG. 3. Immunoelectron microscopy detection of GAPDH in *P. brasiliensis* yeast cells. (A) Transmission electron microscopy of *P. brasiliensis* yeast cells, showing the nucleus (n), intracytoplasmic vacuoles (v), and mitochondria (m). The plasma membrane (arrow) and cell wall (w) are also shown. (B and C) Gold particles (arrowheads) are observed at the fungus cell wall (w) and in the cytoplasm (double arrowheads). (D) Negative control exposed to the rabbit preimmune serum. Bars, 1 μm (A), 0.5 μm (B and D), and 0.2 μm (C).

between *P. brasiliensis* yeast cells and A549 pneumocytes, as shown in Fig. 6. *P. brasiliensis* yeast cells were treated with the antibody anti-GAPDH prior to interaction with the pneumocytes, or pneumocytes were treated with recombinant GAPDH prior to the interaction with *P. brasiliensis*. The controls (nontreated cells) were used to calculate the percentages of both adhesion and infection inhibitions. When *P. brasiliensis* yeast cells were incubated with the anti-GAPDH polyclonal antibody, a decrease of 63% in the adhesion index ($P < 0.05$) was observed to occur. Similarly, when the pneumocytes were treated with recombinant GAPDH the adhesion index was reduced by 68% ($P < 0.05$) compared to the values of nontreated cells (Fig. 6A). Additionally, the infection index was decreased by 80% ($P < 0.0001$) when *P. brasiliensis* yeast cells were treated with the anti-GAPDH polyclonal antibody and by 97% ($P < 0.0001$) when pneumocytes were treated with the recombinant protein (Fig. 6B). Controls were performed by incubating the pneumocytes with BSA prior to the addition of yeast cells (Fig. 6A and B).

DISCUSSION

We have previously identified glycolytic enzymes as host-interacting molecules of *P. brasiliensis* (3, 7, 13, 32). *P. brasiliensis* GAPDH, triose phosphate isomerase, and fructose 1,6-

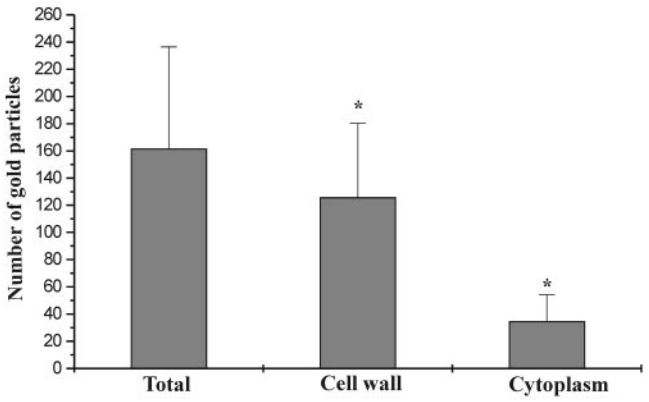

FIG. 4. Mean gold labeling of yeast cells of *P. brasiliensis*. Results, which are representative of five independent preparations, are expressed as the mean gold particles in total cells, cell wall, and cytoplasm. *, P of <0.05 in a comparison of gold labeling in the cell wall and cytoplasm. Vertical bars indicate standard deviations.

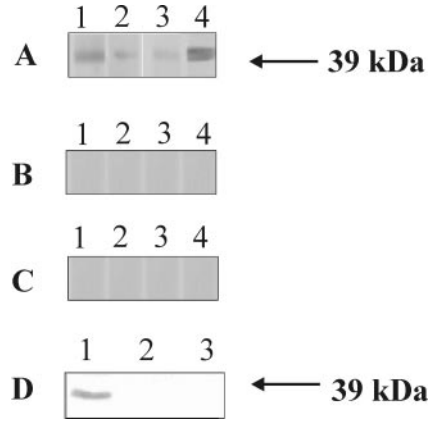

FIG. 5. Binding of *P. brasiliensis* recombinant GAPDH to extracellular matrix components and to pneumocytes in culture. (A) Recombinant GAPDH (0.5 μg) was subjected to SDS-PAGE and electroblotted. Membranes were reacted with laminin (lane 1), fibronectin (lane 2), and type I collagen (lane 3) and subsequently incubated with rabbit IgG antilaminin, antifibronectin, and anti-type I collagen antibodies, respectively. Use of peroxidase-conjugated anti-rabbit IgG revealed the reactions. The positive control was obtained by incubating the recombinant protein with anti-GAPDH polyclonal antibody (lane 4). (B) Recombinant GAPDH (0.5 μg) was subjected to SDS-PAGE and electroblotted. The membranes were reacted with rabbit IgG antilaminin, antifibronectin, and anti-type I collagen antibodies (lanes 1, 2, and 3, respectively). The recombinant protein was incubated just with the secondary antibody, peroxidase-labeled goat anti-rabbit IgG (lane 4). (C) BSA (0.5 μg) was subjected to SDS-PAGE and electroblotted. The membranes were reacted with laminin (lane 1), fibronectin (lane 2), and type I collagen (lane 3) and subsequently incubated with rabbit IgG antilaminin, antifibronectin, and anti-collagen type I antibodies, respectively. Use of peroxidase-conjugated anti-rabbit IgG revealed the reaction. BSA was incubated with anti-GAPDH polyclonal antibody, and the reaction was revealed by using anti-rabbit IgG alkaline phosphatase-coupled antibody (lane 4). (D) Cultured type II pneumocytes were incubated with 50 μg of recombinant GAPDH for 5 h at 37°C. After being washed with PBS to remove the unbound protein, the cells were lysed and the supernatant was fractionated by SDS-PAGE. After transference to membranes, immunodetection was performed by incubation with rabbit anti-GAPDH polyclonal antibody. After incubation with alkaline phosphatase-coupled anti-rabbit IgG, the reaction was developed with BCIP-NBT. Lane 1, supernatant of pneumocytes incubated with recombinant GAPDH; lane 2, pneumocytes not incubated with recombinant GAPDH; lane 3, recombinant protein added to the cell culture flask.

biphosphate aldolase have been described as molecules reactive with sera of patients with PCM. In this context, we previously characterized the cDNA and the genomic sequence encoding GAPDH and demonstrated the protein to be present in larger amounts in the parasitic yeast phase than in the mycelium, suggesting a putative role for GAPDH in the parasitic phase of *P. brasiliensis* (3).

To gain a better understanding of the host-parasite relationships with *P. brasiliensis*, we have demonstrated in this work that the *P. brasiliensis* GAPDH, previously characterized by microsequencing of its N terminus and of endoproteinase Lys-C digested peptides (3, 13), is located at the fungus cell wall. Western blot analysis of the cell extracts that correspond to the most superficial components of the fungal cell wall (5) was initially employed to define the cellular localization of GAPDH at the fungus cell wall. Subsequently, the polyclonal antibody produced against recombinant GAPDH allowed the definition of the protein subcellular localization by immunoelectron microscopy. In addition to its intracellular location, GAPDH was detected at the outermost layer of the cell wall in yeast forms of *P. brasiliensis*, in larger amounts than in the cytoplasm compartment. Cell surface expression of cytosolic enzymes is becoming increasingly recognized on the surfaces of both eukaryotic and prokaryotic cells (16, 30). The presence of GAPDH at the yeast cell surface of *P. brasiliensis* poses interesting questions, such as how its incorporation into the cell wall takes place in the absence of a conventional N-terminal signal sequence responsible for targeting the protein into the classical secretory pathway. In this sense, unusual signal sequences as well as unusual secretory pathways had been proposed for GAPDH. It has been shown that the N-terminal half of the *C. albicans* GAPDH polypeptide encoded by the *TDH3* gene is able to direct its incorporation into the yeast cell wall (9). More studies are needed to identify putative signals related to *P. brasiliensis* cell wall targeting. Another interesting point is the larger amount of gold particles in the cell wall of the yeast form than in the cytoplasm compartment. It has been demonstrated that temperature upshift is one factor that can cause an increase in enzymatically active cell wall-associated GAPDH in *Saccharomyces cerevisiae* (10). The temperature experienced by the yeast cells of *P. brasiliensis* during the infective process has to be investigated to analyze the distribution of GAPDH in this organism.

A critical first step in the establishment of infection by pathogens is the attachment to host components. The recognition of host cells by the pathogen requires the presence of complementary molecules at the surface of the host cells. Some types of adhesins that interact with receptors seem to exist in a number of different pathogens, and host ECM components are of great importance in the modulation of migration, invasion, differentiation, and microbial proliferation. Thus, host proteins such as laminin, collagen, fibronectin, and fibrinogen have been proposed as the microbial cell ligands (31). Several molecules that are present in the ECM may be involved in the adhesion of *P. brasiliensis*. For this reason, it has

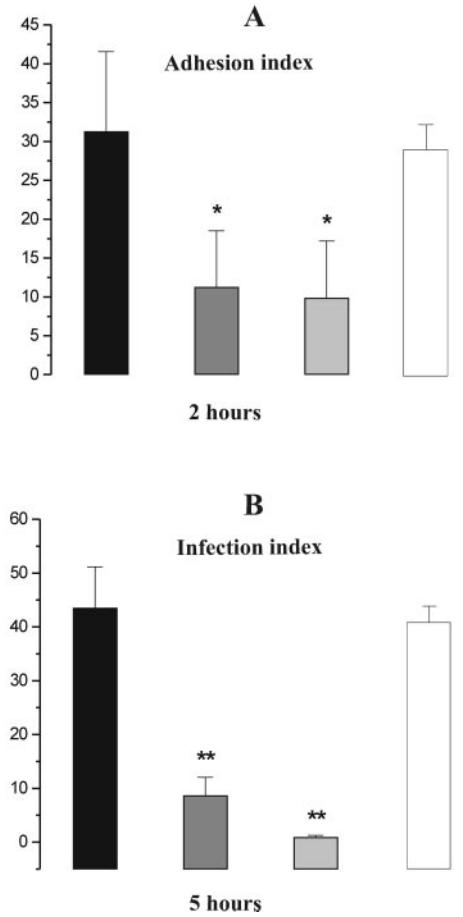

FIG. 6. Interaction of *P. brasiliensis* yeast forms with pneumocytes. Yeast cells were pretreated for 1 h with anti-GAPDH polyclonal antibody (diluted 1:100). In addition, A549 cells were pretreated for 1 h with 25 μg/ml of recombinant GAPDH. As a control, pneumocytes were pretreated for 1 h with 25 μg/ml of BSA. (A) Adhesion of *P. brasiliensis* to pneumocytes was analyzed 2 h after the treatments. (B) Infection (adhesion plus internalization) of *P. brasiliensis* to pneumocytes was analyzed 5 h after the treatments. Black bars, control; dark gray bars, *P. brasiliensis* cells treated with anti-GAPDH polyclonal antibody; light gray bars, pneumocytes treated with recombinant GAPDH; white bars, pneumocytes treated with BSA. The adhesion and infection index values represent the means ± standard deviations of three independent experiments. One asterisk denotes values statistically different from the control ($P < 0.05$), and two asterisks denote significance at a P value of <0.0001. Vertical bars indicate standard deviations.

been known for some time that *P. brasiliensis* exhibits affinity for laminin, fibronectin, and fibrinogen (1, 2, 17, 37).

Here, we introduce the adhesin properties of the cell wall-associated glycolytic enzyme GAPDH. A direct demonstration that surface GAPDH is involved in the interaction of *P. brasiliensis* with laminin, fibronectin, and type I collagen was obtained by adhesion experiments of those molecules to immobilized GAPDH. Laminin and fibronectin are candidate ligands which could mediate adherence of fungal pathogens to host tissues (26, 36). Moreover, epithelial damage caused by pathogens can lead to exposure of subepithelial basement membrane, increasing the accessibility of laminin, type IV collagen, and afterwards that of fibronectin and type I collagen present in the interstitial space to pathogens (6). If the fungus gains access to the intravascular space, it encounters fluid fibronectin. For PCM, it has been described that for the invasive process to start, *P. brasiliensis* has to attach to and pass through the basal membrane to reach blood and lymph capillaries (28). In this context, the binding of GAPDH to laminin, fibronectin, and type I collagen could elect the molecule as a putative virulence factor which could enhance the fungus invasiveness potential.

Studies have demonstrated the capacity of *P. brasiliensis* for adhesion and invasion (2, 22, 27). The GAPDH of *P. brasiliensis* seems to play a role in the early stages of the fungal infection. Both the treatment of pneumocytes with GAPDH and the incubation of *P. brasiliensis* yeast cells with the polyclonal anti-GAPDH antibody resulted in inhibition of adhesion and infection of the epithelial cells by *P. brasiliensis*. The results showed that the presence of the anti-GAPDH antibody resulted in 63% and 80% reductions, respectively, in the ability of *P. brasiliensis* to adhere to and infect pneumocytes. In addition, the treatment of those cells with recombinant GAPDH resulted, respectively, in 68% and 97% reductions in the fungus abilities of binding and infection. All of the data suggest the concept that GAPDH likely plays a role in *P. brasiliensis* adherence and colonization, triggering host cell processes involved in the pathogenesis of this fungal infection. Our data may lead to a better comprehension of *P. brasiliensis* interactions with host tissues and of PCM pathogenesis.

ACKNOWLEDGMENTS

The work was supported by grants from MCT/CNPq and FAPESP. Mônica Santiago Barbosa has a fellowship from CAPES.

We thank Juliana Leal Monteiro da Silva for the helpful comments on the manuscript.

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Editor: A. Casadevall