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## Identification of pH-Regulated Antigen 1 Released from *Candida albicans* as the Major Ligand for Leukocyte Integrin $\alpha_M\beta_2$

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*J Immunol* 2007; 178:2038-2046; ;  
 doi: 10.4049/jimmunol.178.4.2038  
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# Identification of pH-Regulated Antigen 1 Released from *Candida albicans* as the Major Ligand for Leukocyte Integrin $\alpha_M\beta_2$ <sup>1</sup>

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*Candida albicans* is a common opportunistic fungal pathogen and is the leading cause of invasive fungal disease in immunocompromised individuals. The induction of cell-mediated immunity to *C. albicans* is of critical importance in host defense and the prime task of cells of the innate immune system. We previously demonstrated that the integrin  $\alpha_M\beta_2$  (CD11b/CD18) is the major leukocyte receptor involved in *C. albicans* recognition, mediating both adhesive and migratory responses to the fungus. In the present study, we demonstrate that various *C. albicans* strains release a protease-sensitive activity into their conditioned medium that supports  $\alpha_M\beta_2$ -mediated cell adhesion and migration. The isolation and characterization of this protein was undertaken by two independent approaches: 1) immunoaffinity purification on a mAb raised to conditioned medium which blocked  $\alpha_M\beta_2$ -dependent adhesion and migration; and 2) affinity chromatography on purified  $\alpha_M\beta_2$ . Each approach led to the isolation of the same protein, which was unequivocally identified as pH-regulated Ag 1 (Pra1p), based on mass spectrometry and amino acid sequence analyses. *C. albicans* mutant strains lacking Pra1p were unable to support leukocyte adhesion or migration. In a neutrophil-mediated fungal killing assay, such mutant strains were resistant to killing and/or phagocytosis. Addition of purified Pra1p or reagents that block  $\alpha_M\beta_2$  function prevented killing of Pra1p-expressing but not Pra1p-deficient strains of *C. albicans*. Together, these data indicate that Pra1p is a ligand of  $\alpha_M\beta_2$  on *C. albicans* and that the soluble form of Pra1p may assist the fungus in escaping host surveillance. *The Journal of Immunology*, 2007, 178: 2038–2046.

In recent years, fungal endocarditis and arthritis have emerged as important causes of morbidity and mortality in immunocompromised patients (e.g., AIDS, cancer chemotherapy, organ or bone marrow transplantation). Fungal infections account for 20% of all infective endocarditis and have an extremely high mortality rate (70%) (1–4). In addition, hospital-related fungal infections in patients not previously considered at risk (e.g., patients on an intensive care unit) have become a major health concern. *Candida albicans* is the principal causative organism of fungal arthritis and endocarditis. Additionally, *Candida* strains are developing quickly that are resistant to commonly used antimycotics (5, 6). Thus, dissecting its pathogenic mechanisms and the host response to *C. albicans* is of great practical importance.

Both nonspecific and specific immune defenses play roles in protection against disseminated candidiasis. Polymorphonuclear

leukocytes (PMNs)<sup>3</sup> have been shown to be the primary components of the host's innate immune defenses against *Candida* infections in in vitro studies, animal models, and studies of neutropenic patients (7–9). A protective role for macrophages in disseminated candidiasis has also been suggested (10–12). Therefore, a better understanding of the interactions between *C. albicans* and professional phagocytic cells would provide valuable insights into how the host successfully protects itself against such *Candida* infections.

Patients with defects in leukocyte phagocytic functions, such as leukocyte adhesion deficiency (LAD-1), which is characterized by the absence of  $\beta_2$  integrins (CD11/CD18), are highly susceptible to fungal infections, predominantly caused by *C. albicans* (13, 14). One member of this family, integrin  $\alpha_M\beta_2$  (Mac-1, CD11b/CD18, CR3), is the principal adhesion receptor for *C. albicans* on PMNs (15, 16). This cell surface receptor mediates migration of leukocytes to sites of infection, adhesion to fungus, and subsequent killing or phagocytosis of the pathogen (8, 17). As a typical integrin,  $\alpha_M\beta_2$  consists of two noncovalently linked subunits,  $\alpha_M$  and  $\beta_2$ . Previous studies have shown that the recognition of *C. albicans* by  $\alpha_M\beta_2$  depends primarily on its I-domain and its lectin-like domain, both of which reside within its  $\alpha_M$  subunit, and the interaction is modulated by the  $\beta_2$  subunit (16).

The direct interaction of  $\alpha_M\beta_2$  with *C. albicans* has been partially characterized (15, 16, 18, 19); leukocytes use  $\alpha_M\beta_2$  to adhere

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Received for publication August 14, 2006. Accepted for publication November 28, 2006.

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<sup>1</sup> The work carried out in Valencia was partially supported by grants from the European Union (MRTN-CT-2003-504148) and the Spanish Ministerio de Ciencia y Tecnología (BMC2003-01023). Studies performed at the Cleveland Clinic were supported by National Institutes of Health Grant HL66197.

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<sup>3</sup> Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; PVP, polyvinylpyrrolidone; NIF, neutrophil inhibitory factor; Pra1p, pH-regulated Ag 1; CAS, *C. albicans* supernatant; CASL, *C. albicans* soluble ligand for  $\alpha_M\beta_2$ ; RFU, relative fluorescence units.

only to the filamentous form of *C. albicans*, but not to yeast cells. However, the *Candida* hyphal molecules, which are recognized by  $\alpha_M\beta_2$  remain unknown. Even less is known about a soluble chemoattractant activity for leukocytes, which is released from *C. albicans*, other than the demonstration that a *C. albicans* culture filtrate induced migration of both human PMNs and a murine macrophage-like cell line across endothelial cells in an in vitro model (9, 20, 21). Edens et al. (17) also showed that conditioned medium for *C. albicans* induced migration of PMNs and macrophages was stimulated by an unidentified agent that interacted with the formyl peptide receptor on the PMNs, and by an unknown receptor on macrophages. It was also reported that *C. albicans* hyphae release a polypeptide that inhibited the ability of PMNs to bind hyphae (22). Together, these observations provide rationale to suggest that *C. albicans* may release a soluble ligand(s) that is recognized by integrin  $\alpha_M\beta_2$ . The purpose of the present study was to identify and characterize this  $\alpha_M\beta_2$  ligand.

## Materials and Methods

### *C. albicans* strains

Strains 18804, 26555, 36082, 48867, and 56811 were purchased from American Type Culture Collection (ATCC) and routinely maintained on the Sabouraud dextrose agar plates (Difco/BD Biosciences), as well as in frozen stocks. Strains CAS-10 (*phr1Δ/phr1Δ PHR2/PHR2*), CAS-11 (*PHR1/phr1Δ PHR2/PHR2*), and CFM-2 (*PHR1/PHR1 phr2Δ/phr2Δ*) were described previously (23) and maintained on yeast peptone dextrose (Difco) agar plates, supplied with 25  $\mu$ M uridine. Strains SC5314 (clinical isolate) and CAMB43 (*pra1Δ/pra1Δ*), also previously described (24), were maintained either on Sabouraud dextrose agar or yeast peptone dextrose plates at 29°C.

### *C. albicans* supernatant

To prepare a *C. albicans* conditioned medium, 10<sup>8</sup> yeast cells of a selected strain were inoculated into T-175 flasks (Sarstedt), containing 100 ml of high glucose complete RPMI 1640 medium (Invitrogen Life Technologies) and incubated for 2 or 3 days at 30°C (or at 37°C for specific experiments) on an orbital shaker with agitation at 30–40 rpm. After incubation, the fungal cells were removed by centrifugation and the supernatant was clarified by filtration through a 0.22- $\mu$ m filter membrane (Nalgene). This clarified *C. albicans* supernatant is referred to as “CAS” and was used immediately for assays. In some cases, the CAS was concentrated 10- to 1000-fold using an Amicon Centricon ultrafiltration centrifugal device with a 3-kDa cutoff limit (Millipore). For enzyme treatment, 1-ml aliquots of CAS were incubated with either 5 U of glycopeptidase F (PNGase), 2  $\mu$ g of pronase, 10 U of trypsin, 10  $\mu$ g of  $\alpha$ -amylase, 1000 U of  $\beta$ -amylase, or 10  $\mu$ g of *Vibrio cholerae* neuraminidase for 7 h at 37°C and then 100 U of soybean trypsin inhibitor, 10  $\mu$ g of neuraminidase inhibitor (zanamivir), or 10 mg of amylase inhibitors (all enzymes and inhibitors were from Sigma-Aldrich) were added to the relevant enzyme mixtures. After addition of the inhibitors, samples were incubated for 1 h at room temperature, then overnight at 4°C, and used next day. As controls, RPMI 1640 with no CAS but containing the various enzymes and inhibitors was used. For pronase, we relied on its inactivation by autodegradation, which occurs within 2–4 h (25), and no residual amidolytic activity using Benzoyl-arginine-pNA as a substrate was detected.

### Cells

THP-1 monocytoid cells were obtained from ATCC and maintained in complete RPMI 1640 supplemented with 20% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Human epithelial kidney cell line HEK293 stably transfected with  $\alpha_M\beta_2$  described previously (16, 26, 27) was maintained in DMEM/F-12 medium (Invitrogen Life Technologies), containing 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. These cells also were used as a source for  $\alpha_M\beta_2$  purification (see below).

Human neutrophils were isolated from peripheral blood of healthy volunteers drawn into sterile acid-citrate dextrose (one-seventh volume of 145  $\mu$ M sodium citrate (pH 4.6) and 2% dextrose). Isolation was performed by density gradient centrifugation on Ficoll-Hypaque (Amersham Biosciences), followed by dextran sedimentation of erythrocytes and hypotonic lysis of residual erythrocytes (28).

### Monoclonal Abs

The following previously characterized mAbs to  $\alpha_M\beta_2$  were used: 44a (anti- $\alpha_M$  integrin subunit I-domain), OKM1 (anti- $\alpha_M$  integrin subunit lectin-like domain), IB4 (anti- $\beta_2$  integrin subunit), and W6/32 (anti-MHC class I). The hybridoma cell lines producing these mAbs were obtained from the ATCC and the mAbs were purified from mouse ascites using recombinant protein G columns (Zymed Laboratories).

To develop anti-CAS mAbs, BALB/c mice (6 wk old, female) were immunized with 1000-fold concentrated CAS from *C. albicans* strain 36082 in CFA (Pierce), followed with further immunizations every 4 wk with 1000-fold concentrated CAS obtained from strains 26555, 48867, 56811, and again 36082 (in this order). The last immunization was performed 3 days before harvest of spleen cells and their fusion with mouse myeloma P3.X63.Ag8.U1 cells. Hybridoma supernatants were screened for their ability to block HEK293/ $\alpha_M\beta_2$  cell adhesion to CAS adsorbed on plastic (see *Cell adhesion assays* below). Hybridomas producing blocking mAbs were then cloned by limiting dilution. Ultimately, 11 hybridoma clones producing cell adhesion blocking mAbs were established.

### $\alpha_M\beta_2$ purification

$\alpha_M\beta_2$  was purified from transfected HEK293 cells using a modification of the method of Miller et al. (29) as described from our laboratory. Briefly, 10 g of HEK293/ $\alpha_M\beta_2$  cells were harvested, washed, and lysed with 20 ml of 1% Triton X-100 in 0.15 M NaCl, 0.05 M Tris (pH 7.3) (TBS), containing 0.5 ml of protease inhibitor mixture for mammalian cells (Sigma-Aldrich). The cell lysate was clarified by centrifugation, diluted 4-fold with TBS, and loaded onto a 1  $\times$  4 cm column containing IB4 mAb agarose at 4°C (1.8 mg of IB4/ml of CNBr-activated Sepharose 4B (Amersham)). After washing with TBS containing 10 mM octyl- $\beta$ -D-glucopyranoside (Sigma-Aldrich) and 1 mM CaCl<sub>2</sub>, bound protein was eluted with 20 mM sodium acetate buffer (pH 4.2) containing 10 mM octyl- $\beta$ -D-glucopyranoside. Immediately after elution, the pH of the eluates was adjusted to pH 7.2 with 1 M Tris. Protein concentration of the purified material was determined by BCA assay from Pierce. The  $\alpha_M\beta_2$  purified by this protocol has been characterized by us previously (30, 31).

### Cell adhesion assays

For cell adhesion to CAS, 48-well Costar tissue-culture plates (Corning) were coated overnight at 4°C with 200  $\mu$ l of different dilutions (0- to 50-fold) of CAS and then postcoated with 0.5% polyvinylpyrrolidone (PVP) for 1 h at room temperature. Control wells were coated with PVP only. Before use, the plates were rinsed three times with PBS. For direct cell adhesion to *C. albicans*, aliquots of 5  $\times$  10<sup>5</sup> *C. albicans* blastoconidia in RPMI 1640 medium, containing 1% FBS, were incubated in 48-well tissue-culture plates (Corning) overnight at 37°C to germinate, and then washed three times with HBSS/HEPES and used for adhesion without PVP coating (16, 18). Before plating for germination, the *Phr1<sup>mut</sup>* *C. albicans* strain was initially incubated in RPMI 1640 medium, adjusted to pH 5.5 with 1 M HEPES for 6 h at 37°C (32); then the acidic medium was removed, fresh RPMI 1640 (pH 7.4) was added, and the fungus was incubated as described above for other strains. Before experiments, germination of this and other *C. albicans* strains was confirmed by microscopy; at use, no bud forms were detected and >95% of all yeast cells were in a mycelial form. THP-1 and HEK 293 cells were harvested using HBSS-based enzyme-free Cell Dissociation Buffer (Invitrogen Life Technologies), harvested as described above, washed three times with 50 ml of divalent ion-free HBSS/HEPES (pH 7.4), and resuspended in the HBSS/HEPES, containing 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, and 5% BSA. The cells were then seeded at 1.5–2  $\times$  10<sup>5</sup> cells/well onto the assay plates and incubated at 37°C for 30 min. In inhibition experiments, the cells were pretreated with the selected mAbs or other reagents for 15 min at 37°C before addition to the coated wells. To determine the extent of adhesion/attachment, the plates were washed three times with PBS, and the number of adherent cells in each well was quantified using the Cyquant Cell Proliferation Assay kit (Molecular Probes) according to the manufacturer's instructions and as previously described. Data from cell adhesion and migration assays (see below) are presented as relative fluorescence units (RFU) and represent the results of three independent experiments.

### Cell migration assays

Cell migration assays were performed in serum free DMEM/F-12 medium (Invitrogen Life Technologies) using Costar 24-transwell plates with tissue-culture treated 8- $\mu$ m pore polycarbonate filters (Corning) as previously described (30, 33). The lower chambers contained 600  $\mu$ l of medium with

varying dilutions of the CAS, purified protein or  $5 \times 10^5$  germinated *C. albicans* blastoconidia, prepared as described above. The upper chambers contained final volumes of 200  $\mu$ l of cell suspension. The assay was initiated by addition of 50  $\mu$ l of cell suspension ( $2 \times 10^5$  cells/well) to the medium in the upper chambers, and the plates were placed in a humidified incubator at 37°C, 5% CO<sub>2</sub> for 16 h. The migrated cells, present on the undersurface of the membrane, as well as in the lower chamber, were quantitated using the Cyquant Cell Proliferation kit as described above and previously (30).

#### Neutrophil candidacidal assay

*C. albicans* phagocytosis and killing by human neutrophils were quantified by a modification of the method of Lehrer et al. (8). Briefly,  $10^6$  *C. albicans* blastoconidia in 0.25 ml of high glucose RPMI 1640, containing 5% FBS, were allowed to germinate in  $12 \times 75$ -mm plastic tubes at 37°C for 1 h with agitation. The *C. albicans* cells were collected by centrifugation, washed twice with 3 ml of HBSS/HEPES (pH 7.4), suspended in 0.25 ml of HBSS/HEPES, and mixed with 0.25 ml of neutrophil suspension containing  $3 \times 10^6$  cells. The leukocyte/yeast mixture was incubated at 37°C with slow shaking for 1–5 h. Next, 0.25 ml of 2.5% sodium deoxycholate (Sigma-Aldrich) in HBSS/HEPES was added, the mixtures were briefly vortexed, and the lysed leukocytes were removed by centrifugation. The *C. albicans* cells were resuspended in 0.25 ml of 2.5 mM methylene blue (Sigma-Aldrich) in HBSS/HEPES, and the number of viable cells was counted in a hemacytometer using a microscope. Control samples contained *C. albicans* incubated without neutrophils.

#### Mass spectrometry

Mass spectrometry was performed as described in (34). Briefly, protein samples were separated by SDS-PAGE, and proteins were visualized with Coomassie G-250. The protein bands were cut from the gel, washed, possible disulfide bonds were reduced with DTT and alkylated with iodacetamide, and the gel pieces were incubated overnight at room temperature with 20 ng/ $\mu$ l trypsin. The generated peptides were extracted from the polyacrylamide gel with 50% acetonitrile/5% formic acid, separated on 10 cm  $\times$  75  $\mu$ m Phenomenex Jupiter C18 reversed-phase capillary column (Phenomenex) and analyzed using a Finnigan LCQ-Deca ion trap mass spectrometer system (Thermo Electron). The digest was analyzed using the data-dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular masses and product ion spectra to determine amino acid sequences in a single instrument scan. All matching spectra were verified by manual interpretation. Some samples also were analyzed by MALDI-TOF using a Micromass ToFSpec 2E time-of-flight mass spectrometry system (Micromass).

#### Protein sequencing

Protein samples were separated by SDS-PAGE on 4–20% gradient gels (Bio-Rad) and transferred electrophoretically onto polyvinylidene difluoride membranes. The N-terminal amino acid sequence was determined by Edman degradation chemistry using a Procise 492 Protein sequencer (Applied Biosystems).

#### Data analysis

The data are expressed as means  $\pm$  SEM. To determine the significance of differences between two groups, a two-tailed Student's *t* test was performed using the Sigma-Plot software program (SPSS); *p* < 0.05 was considered significant.

## Results

The prior studies of Cutler (20), Geiger et al. (21), and Edens et al. (17) showing that *C. albicans* releases a leukocyte chemoattractive activity and our own data showing that integrin  $\alpha_M\beta_2$  is the principal adhesion receptor on leukocytes for *C. albicans* (15, 16) suggested that *C. albicans* might release an  $\alpha_M\beta_2$  ligand. We began to explore this possibility by collecting the conditioned medium (CAS) from several commercially available *C. albicans* strains and comparing their ability to support adhesion and migration of  $\alpha_M\beta_2$  expressing THP-1 monocytoid cells. As shown on Fig. 1A, when immobilized on microtiter wells, CAS from all the *C. albicans* strains tested supported THP-1 adhesion. The adhesive activity accumulated over time and supported a maximal adhesive response within 3–4 days. CAS were harvested from all strains after 3 days in culture and various dilutions were tested for their ability

to induce THP-1 migration. As shown in a Fig. 1B, CAS from all *C. albicans* supported migration in a dose-dependent manner. For subsequent experiments, CAS from *C. albicans* strain 36082 was used, as it was the most effective in supporting leukocyte adhesion and migration.

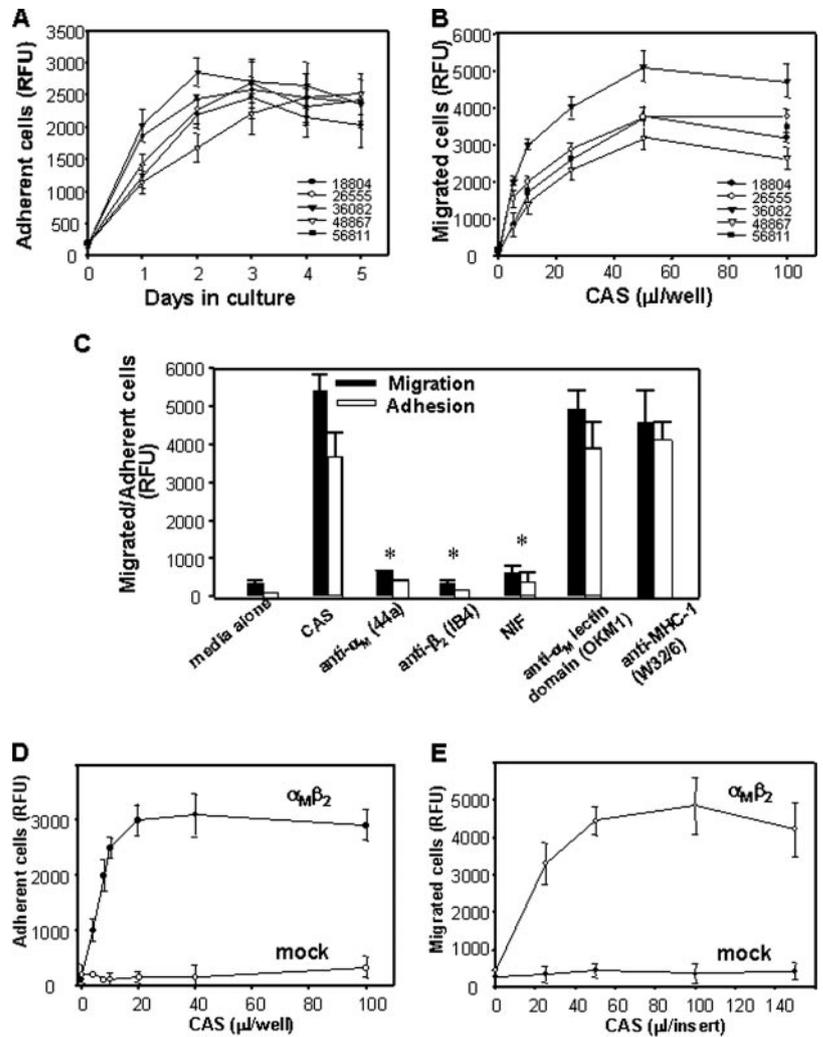
Both THP-1 adhesion and migration to this CAS were completely abrogated by a high-affinity ligand of  $\alpha_M\beta_2$ , neutrophil inhibitory factor (NIF), and by function blocking mAbs to either subunit, anti- $\alpha_M$  (44a) and anti- $\beta_2$  (IB4), of this integrin, but not by a control mAb to MHC-1 (W32/6) or by a mAb, OKM-1, to the lectin domain within the  $\alpha_M$  subunit (35) (see Fig. 1C). In addition, HEK293 cells expressing  $\alpha_M\beta_2$  on their surface, but not mock-transfected cells, showed dose-dependent and  $\alpha_M\beta_2$ -dependent adhesion and migration to CAS (Fig. 1, D and E, respectively).

The results above suggest that a product released from *C. albicans* supports leukocyte adhesion and migration via interaction with  $\alpha_M\beta_2$ , and the nature of this ligand was sought. The sensitivity of the migratory and adhesive activity to several peptidases, lipases, and amylases was tested. CAS was pretreated with pronase, trypsin, the glycosidase, PNGase,  $\alpha$ - and  $\beta$ -amylases, lipase and phospholipase A, and the effects of these treatments on the adhesion and migration of neutrophils were tested (Fig. 2). Treatment of CAS with pronase or trypsin completely abolished the ability of CAS to support leukocyte adhesion and migration, suggesting that the activity of CAS contained peptide bonds, while treatment with amylases, neuraminidase, PNGase (see Fig. 2), or lipase and phospholipase A (results not shown) had no effect.

Concentrated (1000-fold) CAS from three *C. albicans* strains (26555, 36082, and 48867) was used to immunize mice with the goal of obtaining mAbs that blocked its adhesive and migratory activity. The supernatants obtained from hybridoma clones were tested for their ability to block adhesion of HEK 293  $\alpha_M\beta_2$  cells to CAS. Altogether, 11 mAbs with blocking activity were identified. The most potent blocking mAb, CAS-5 (IgM,  $\kappa$ ), was used in an attempt to immunopurify the activity within a CAS. Concentrated CAS was passed over a column with CAS-5 mAb immobilized on agarose beads and the bound protein was eluted at low pH. Both starting CAS and the low pH eluate from the CAS-5 column supported adhesion and migration of HEK 293  $\alpha_M\beta_2$  cells (Fig. 3A). In contrast, the pass-through fraction containing materials that did not interact with CAS-5 had little residual activity. The eluate was characterized by SDS-PAGE and Western blotting (Fig. 3, B and C). When the sample was prepared for gel analysis at a temperature of <40°C, a single major Coomassie blue staining band of ~250 kDa was observed. Upon heating of the sample at 65°C for 10 min or 100°C for 2 min, the 250-kDa band disappeared and several bands with lower molecular masses were observed (Fig. 3B). When these samples were analyzed by Western blotting using the CAS-5 mAb, a single reactive band at 52 kDa was observed in the boiled samples. A faint reactivity at ~250 kDa (due to poor transfer out of gel) was detected in the unboiled samples (Fig. 3C). Of the 11 blocking mAbs, all reacted with the 52-kDa band in Western blots (data not shown).

Mass spectrometry of tryptic digests was used to identify both the 250- and 52-kDa protein bands. As shown in Table I, the masses of 11 tryptic peptides from the 52-kDa protein band were all consistent with derivation from a single *C. albicans* protein, pH-regulated Ag 1 (Pra1p) encoded by *PRA1* (24), which has also been identified as the fibrinogen-binding mannoprotein (36). Additionally, mass-spectrometry analysis of a tryptic digest of the 250-kDa band demonstrated the presence of Pra1p (the same 11 peptides as in 52-kDa band were identified) as well as two other

**FIGURE 1.** CAS supports leukocyte adhesion and migration via integrin  $\alpha_M\beta_2$ . *A*, THP-1 monocytoid cell adhesion to CAS. Tissue-culture plates were coated for 16 h at 4°C with 200  $\mu$ l of 10-fold diluted conditioned medium collected at the indicated times from various strains of *C. albicans* and prepared as described in *Materials and Methods*. Plates were postcoated with 0.5% PVP for 1 h at room temperature and  $1.5\text{--}2 \times 10^5$  THP-1 cells/well were allowed to adhere for 30 min at 37°C. After three washings with PBS, the adherent cells were quantified using the Cyquant Cell Proliferation Assay kit. *B*, THP-1 migration to CAS. Cell migration was measured in Boyden chambers in serum-free DMEM/F-12 medium. THP-1 cells at  $2 \times 10^5$ /well were added to the upper chambers, while lower chambers contained various dilutions of CAS in a total volume of 600  $\mu$ l. Plates were incubated for 16 h in a humidified incubator at 37°C and 5% CO<sub>2</sub>. The migrated cells were counted using the Cyquant Cell Proliferation kit. *C*, THP-1 cells were pretreated with function blocking mAbs to  $\alpha_M\beta_2$ , control mAb to MHC-1 at 20  $\mu$ g/ml, or NIF (20 nM) for 15 min at 37°C and subsequently used in adhesion and migration assays. The 3-day CAS from *C. albicans* strain 36082 was diluted 10-fold before use. The asterisks indicates statistical significance ( $p < 0.01$ ) compared with control. *D* and *E*, HEK 293 cells expressing  $\alpha_M\beta_2$  integrin or mock-transfected cells were added to CAS-coated plates or Boyden chambers at  $2 \times 10^5$ /well, and their adhesion (*D*) and migration (*E*) were quantified. The data are the means  $\pm$  SEM of triplets from three independent experiments.



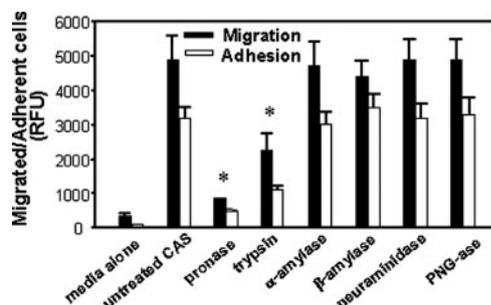
*C. albicans* proteins (see Table I). Eight peptides matched the mannoprotein MP65 sequence (37) (NCB no. 12057031, 39.3 kDa), and 2 peptides matched the 93.7 kDa hyphally regulated protein precursor (38) (accession no. P4691).

Because  $\alpha_M\beta_2$  is directly involved in the leukocyte-fungus interaction, an independent approach to purification of CASL was designed based upon this recognition. Initially, we sought to purify the activity using recombinant  $\alpha_M$  I-domain, a recognition site for

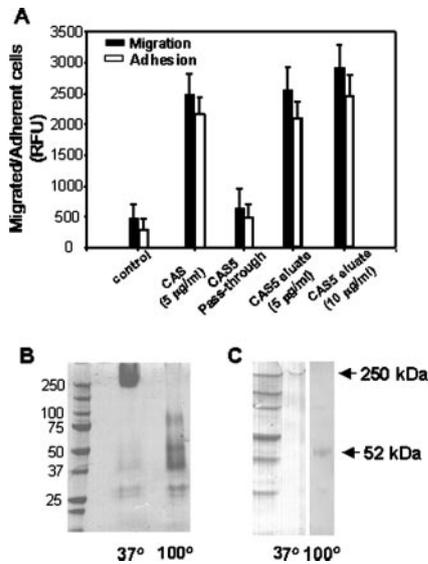
many  $\alpha_M\beta_2$  ligands (39, 40), including *C. albicans* (16), immobilized on agarose beads. However, yields were low and variable. Hence, purification of the activity was undertaken on isolated  $\alpha_M\beta_2$ . Recombinant receptor was isolated as previously described (41), biotinylated, and immobilized on streptavidin-agarose. CAS was passed over the affinity column in the presence of 2 mM Ca<sup>2+</sup>/Mg<sup>2+</sup> and 10 mM *n*-octyl glucopyranoside, and bound proteins were eluted with HBSS-based Enzyme-Free Cell Dissociation Buffer (Invitrogen Life Technologies), containing 10 mM octyl-glucopyranoside. The eluate was analyzed by SDS-PAGE and tested for its ability to support THP-1 cell adhesion and migration.

SDS-PAGE of the eluted sample is shown in Fig. 4A. Only one major protein band was detected, which had a molecular mass of ~68–70 kDa. The NH<sub>2</sub>-terminal sequence of the protein was determined by conventional sequencing and found to be APVXVXRFVDASPXGY, which corresponds to residues A<sub>16</sub>PVTVTRFVDASPTGY<sub>31</sub> of *C. albicans* Pra1p/Fbp1p precursor, and to the NH<sub>2</sub>-terminal sequence of mature Pra1p (24, 36). Mass-spectrometry of tryptic-digested sample identified 10 of the same 11 peptides obtained from the mAb CAS-5 column, all derived from the Pra1p, and these accounted for >35% of the total Pra1p sequence.

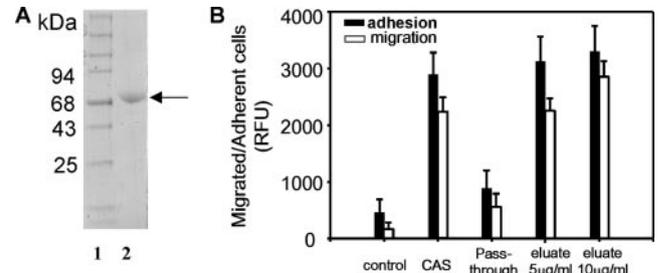
When the ability of THP-1 cells to interact with Pra1p in the  $\alpha_M\beta_2$  column eluate was tested, robust adhesion was observed (see Fig. 4B). This activity was observed in the starting CAS applied to



**FIGURE 2.** The adhesive and migratory activity in CAS is protein. CAS derived from a 3-day culture of *C. albicans* strain 36082 was incubated with the indicated enzymes for 7 h at 37°C at concentrations specified in *Materials and Methods*, and then assessed for its capacity to support adhesion or migration of human PMNs. The results are means  $\pm$  SEM from triplicate trials of three independent experiments. \*, Statistical significance ( $p < 0.01$ ) compared with control.



**FIGURE 3.** Isolation of the  $\alpha_M\beta_2$  ligand from CAS by affinity chromatography on mAb CAS-5. **A**, THP-1 cell adhesive and migratory activities of fractions obtained from a mAb CAS5 agarose column. The fractions are: 1) starting CAS, diluted 10-fold; 2) the unbound fraction, pass-through, from the CAS-5 column; and 3) the low pH eluate from the CAS-5 column. **B** and **C**, SDS-PAGE analysis of the eluate from the CAS-5 mAb column. **B**, The low pH eluate from the CAS-5 mAb-agarose column was analyzed on 10% polyacrylamide gels with sample preparation either at 37°C (lane 2) or 100°C (lane 3) before loading on the gel under reducing conditions. The gels were stained with Gel-Blue B and lane 1 displays the molecular mass markers. **C**, The proteins were transferred onto nitrocellulose membranes, probed with the CAS-5 mAb, and reactivity was developed with HRP-conjugated goat anti-mouse IgG and the Opti-4CN Substrate kit.



**FIGURE 4.** Biochemical and functional analysis of proteins purified from CAS on an  $\alpha_M\beta_2$ -affinity column. **A**, SDS-PAGE analysis of fraction eluted from the  $\alpha_M\beta_2$ -agarose column. Lane 1 was loaded with molecular mass markers and lane 2 is the cell dissociation buffer eluate, which was concentrated and applied onto a 10% acrylamide gel under reducing conditions (the sample was prepared at 37°C). Proteins are detected with Gel-Blue Code. **B**, Adhesion and migration of THP-1 cells to fractions from the  $\alpha_M\beta_2$ -agarose column. The fractions were: 1) the starting CAS, diluted 10-fold; and 2) the pass-through from the  $\alpha_M\beta_2$  column and the cell dissociation buffer eluate from the  $\alpha_M\beta_2$  column at two different concentrations.

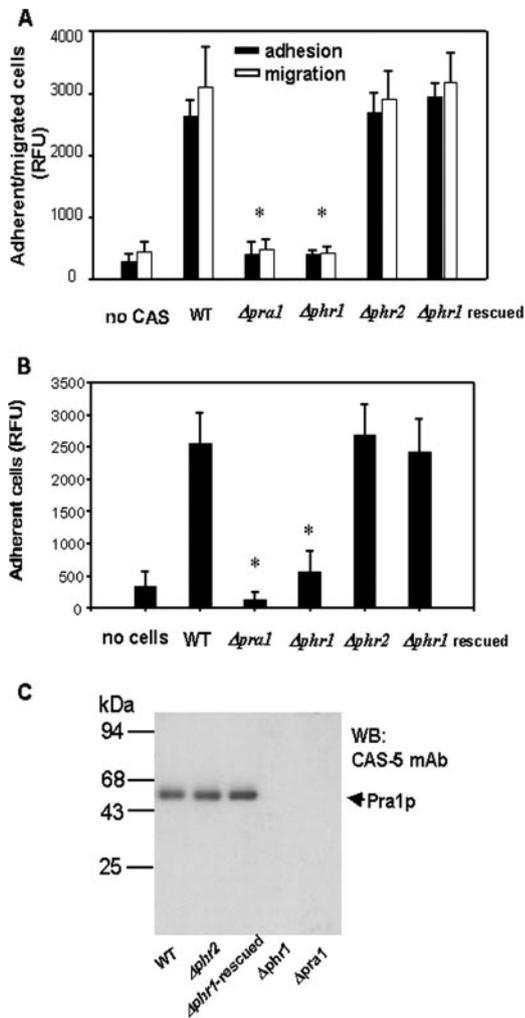
the column and was depleted from the column pass-through. We next tested the capacity of purified Pra1p to support cell migration. As shown on Fig. 4B, Pra1p induced robust and concentration-dependent THP-1 migration.

To verify the importance of Pra1p as an  $\alpha_M\beta_2$  ligand, we used mutant strain of *C. albicans*, in which the *PRA1* gene had been disrupted (24). As shown on Fig. 5A, CAS derived from this  $\Delta pra1$  strain supported neither adhesion nor the migration of THP-1 cells. Additionally, the  $\Delta pra1$  *C. albicans* strain itself also failed to support adhesion of THP-1 cells (Fig. 5B). Although  $\Delta pra1$  *C. albicans* are defective in germination and hyphal formation at temperatures above 42°C, they do form hyphae at 37°C (42), the temperature in which the cells were cultured and the CAS was prepared from

Table I. Results of mass-spectrometry analysis of the peptides<sup>a</sup>

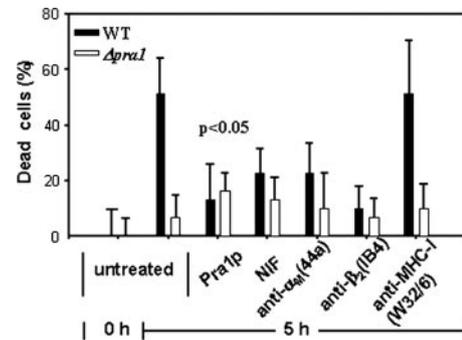
Molecular Mass (obtained)	Molecular Mass (database)	Protein Sequence	Database Sequence
<i>C. albicans</i> Pra1p (from both 250- and 52-kDa bands)			
618.3	618.7	(35–39)	ADWVK
653.3	653.7	(80–84)	SPFFR
730.4	730.9	(180–184)	FWHLK
1124.4	1125.2	(124–132)	NDGWAGYWR
1412.6	1413.6	(122–132)	CKNDGWAGYWR
1413.6	1414.5	(23–34)	FVDASPTGYDWR
1442.7	1443.6	(168–179)	TNIFWAGDLLHR
1540.7	1541.7	(109–121)	SSILFLCDDLDDK
1562.7	1563.7	(159–165)	YLSQLCSGGYTVSK
1718.8	1719.9	(151–165)	RYLSQLCSGGYTVSK
1828.8	1830.0	(109–123)	SSILFLCDDLDDKCK
<i>C. albicans</i> mannoprotein MP65 (250-kDa band)			
892.4	893.0	(303–310)	VWTACGGK
902.4	902.9	(373–379)	YWGIIYSN
1030.5	1031.1	(324–334)	GDSNGVAVPSK
1146.6	1147.2	(335–345)	SNQQAIISSIK
1372.7	1373.5	(312–323)	NVLITETGWPSR
1532.6	1533.6	(128–141)	GITYSPYSDNGGCK
2001.9	2003.2	(346–363)	SSCGASAILFTAFNDLWK
2354.2	2355.7	(182–204)	IFAGIFDVSITSCIESLAEAVK
Hyphally regulated protein precursor (250-kDa band)			
735.4	735.9	(135–142)	STAYLYAR
944.4	945.0	(67–73)	GASLFIK

<sup>a</sup> Results of mass-spectrometry analysis of the peptides obtained upon trypsin digestion of the 250- and 52-kDa band purified from the *C. albicans* CAS using a mAb CAS-5 affinity column.



**FIGURE 5.** Pra1p is required to support adhesion and migration of THP-1 cells. *A*, CAS was collected from the indicated *C. albicans* mutant strains and tested for their capacity to support the adhesion and migration of THP-1 cells as described in Fig. 1. The data are means  $\pm$  SEM from three experiments, each performed in triplicate. \*, Statistical significance for adhesion ( $p < 0.01$ ) and migration ( $p < 0.05$ ) compared with control. *B*, Adhesion of THP-1 cells directly to various *C. albicans* strains. The *PHR1*<sup>null</sup> mutant was allowed to germinate at pH 5.5 to induce hyphal formation. *C*, Western blot of unfractionated CAS collected from culturing the indicated *C. albicans* strains for 4 days. Proteins in the CAS were concentrated  $\times 10,000$ , the samples were heated to 100°C for 2 min under reducing conditions, separated in the gel with 8–16% concentration gradient of polyacrylamide, transferred to nitrocellulose membranes, and probed with CAS-5 mAb as described in Fig. 3. \*, Statistical significance ( $p < 0.05$ ) compared with control.

these cells. The presence of hyphae was verified by microscopic examination of the cultured  $\Delta pra1$  *C. albicans*. Independent evidence for the role of Pra1p as an  $\alpha_M\beta_2$  ligand also was obtained using two other mutant *C. albicans* strains with deletions of either the *PHR1* or *PHR2* genes. The expression of the Phr1p and Phr2p proteins is pH-dependent: Phr1p is expressed on *C. albicans* at neutral pH and Phr2p is expressed at acidic pH (32, 43). The *PHR2*<sup>null</sup> mutant is able to form hyphae at neutral pH; germination by the *PHR1* null-strain is defective at this pH but can produce hyphae at slightly acidic pH (32). These growth condition maneuvers were used to obtain hyphal forms of both *C. albicans* strains and the presence of hyphae was verified microscopically. From the literature, it is known that *PRA1* expression is dependent upon *PHR1* but is not influenced by *PHR2* (44).



**FIGURE 6.** Pra1p mediates  $\alpha_M\beta_2$ -dependent killing of *C. albicans* by human PMNs. *C. albicans* blastoconidia ( $10^6$ ) were allowed to germinate at 37°C for 1 h, pelleted by centrifugation, washed twice with HBSS/HEPES buffer and mixed with  $3 \times 10^6$  freshly isolated PMNs. After incubation for 5 h at 37°C, the PMNs were lysed with 2.5% sodium deoxycholate. *C. albicans* cells were stained with 2.5 mM methylene blue. Dead cells were counted in a hemacytometer under a microscope. Control samples were lysed immediately after PMNs addition without incubation (0 h) and were assigned a value of 0% dead cells. Results are expressed as means  $\pm$  SEM from three experiments performed with PMNs isolated from three different donors. The Pra1p and NIF were used at 2  $\mu$ g/ml and Abs at 20  $\mu$ g/ml final concentration.

Consistent with the identity of Pra1p as the soluble  $\alpha_M\beta_2$  ligand, conditioned medium from a *C. albicans* strain with a disrupted *PHR1* gene failed to support THP-1 cell adhesion and migration (Fig. 5*A*). The capacity to support THP-1 cell adhesion and migration was restored in a “rescued” strain in which the *PHR1* gene was reinserted into a  $\Delta phr1$  mutant. In contrast, CAS from a  $\Delta phr2$  strain, in which *PRA1* expression is not affected, supported the responses of the cells. Western blots of the conditioned medium of these various *C. albicans* strains with mAb CAS-5 supported these interpretations (Fig. 5*C*). The mAb showed reactivity with the conditioned medium derived (4-day cultures concentration 1000-fold) from wild type, *PHR1* rescued cells and  $\Delta phr2$  cells, but not with that from  $\Delta pra1$  or  $\Delta phr1$  cells. When the mutant strains were tested for THP-1 adhesion directly to the fungus, only *PRA1*- and *PHR1*-null mutants failed to bind THP-1 cells, whereas *PHR2*<sup>null</sup> and “rescued” *PHR1* mutant strains supported adhesion to an extent similar to wild-type *C. albicans* (Fig. 5*B*).

Because the  $\beta_2$  integrins are critical receptors in mediating pathogen killing and phagocytosis (45–48), the role of *PRA1* in the  $\alpha_M\beta_2$ -dependent killing of *C. albicans* by human PMNs was evaluated (Fig. 6). Isolated peripheral blood PMNs were added to wild-type and  $\Delta pra1$  strains at a ratio of 3:1. At time zero, >98% of the cells of strains were viable as assessed by methylene blue staining. In the absence of PMNs, the viability of the wild type and  $\Delta pra1$  strains remained high, only 7 and 5% of the *C. albicans* were not viable, respectively, at a 5-h time point. At 5 h of incubation with PMNs, however, only 52% of the wild-type cells remained alive as contrasted to 90% viability of the  $\Delta pra1$  strain. However, this increased susceptibility of the wild-type control strain was reversed when the incubations were performed in the presence of 2  $\mu$ g/ml purified Pra1p; the viability remained >85% ( $p < 0.05$ ). In contrast, the  $\Delta pra1$  strain showed little change in viability in the presence of purified Pra1p (88 and 81% with and without added Pra1p, respectively). In addition, reagents that block the function of  $\alpha_M\beta_2$ , NIF and receptor-specific mAbs, but not the control anti-MHC-1 mAb, significantly suppressed PMN-mediated killing of the wild-type strains (78–90% viability). These reagents had no effect on killing of the  $\Delta pra1$  strain.

## Discussion

In the present study, we identify Pra1p as a major *C. albicans* ligand of leukocyte integrin  $\alpha_M\beta_2$ . Our data further indicate that Pra1p is recognized by the integrin not only as a component of *C. albicans* cell wall but also as a soluble ligand released from the fungus. Two independent approaches led to the identification of Pra1p as the soluble  $\alpha_M\beta_2$  ligand released from *C. albicans*. In the first approach, CAS was used to prepare mAbs that blocked adhesion and migration of THP-1 cells to CAS. All of these mAb recognized on immunoblots a 52-kDa protein within CAS and one of these was used to immunopurify and characterizes its target Ag. By mass spectrometry, the target Ag was identified as Pra1p. In the second approach,  $\alpha_M\beta_2$  was used to purify cognate ligands within CAS. Again, a single major band was isolated and was again identified as Pra1p by N-terminal sequencing and mass spectrometry. The convergence of these two approaches on a single molecule provides strong evidence that Pra1p is the major soluble ligand of  $\alpha_M\beta_2$  released from *C. albicans*.

Pra1p is a mannoprotein and its carbohydrate content can contribute as much as 25% of its mass. Previously, Lopez-Ribot et al. (36) and Polonelli et al. (49) noted that the molecular mass of Pra1p from different *Candida* strains varied and suggested that this heterogeneity was due to differences in protein glycosylation (50). Because the sequences of the  $\alpha_M\beta_2$  ligand isolated by the two approaches unequivocally identified it as Pra1p, differences in posttranslational modifications may account for the variability in the molecular mass of the Pra1p we isolated, 68–70 or 52 kDa. Such modifications, which could modify the apparent molecular mass of Pra1p include glycosylation or ubiquitination (51). It is conceivable that such modifications could lead to preferential recognition of a particular form of Pra1p by  $\alpha_M\beta_2$ . Also partial proteolysis may not be excluded; a peptide containing the N-terminal sequence was obtained from the 52-kDa form of Pra1p, but no tryptic peptide corresponding in mass of the predicted C-terminal peptide of Pra1p was obtained. Additional peptides were detected in the mass spectral analyses of Pra1p, but their sequences could not be deduced, and these may represent glycopeptides derived from Pra1p, including a C-terminal peptide. Also, the failure to identify the predicted threonine residues at positions 4 and 6 of the mature Pra1p (Table I) is likely to contain posttranslational modifications.

mAbs to both the  $\alpha_M$  (mAb 44a) and  $\beta_2$  (mAb IB4) subunits inhibited Pra1p recognition by  $\alpha_M\beta_2$ ; these mAbs inhibit interaction of many protein ligands to  $\alpha_M\beta_2$  (27, 52). Multiple ligands use the  $\alpha_M$ I-domain to their engagement of the integrin (53, 54) and NIF, which binds selectively to I domain within the  $\alpha_M$  subunit (26), also was a potent inhibitor of Pra1p-containing CAS. We have previously reported that mAb OKM-1 to the lectin-like domain in the  $\alpha_M$  subunit and certain sugars were weak/partial inhibitors of recognition of intact *C. albicans* by the integrin (16). In contrast, OKM-1 had no apparent effect on Pra1p recognition by  $\alpha_M\beta_2$ -expressing cells (Fig. 1C). Furthermore, treatment of CAS with carbohydrases had no effect on activity while treatment with proteases, which cleave the protein backbone but would retain glycopeptides within the digests, destroyed activity. Together, these results suggest that the lectin-like domain may contribute weakly to recognition of *C. albicans* recognition by  $\alpha_M\beta_2$ , but does not play a substantial role in its recognition of released forms of Pra1p. The possibility that there is a requirement for sequential engagement of Pra1p protein followed by a low affinity recognition of a particular Pra1p carbohydrate moiety by the lectin-like domain cannot be excluded.

It was originally reported that deletion of the *PRA1* gene suppressed germination of *C. albicans*; However, this effect was only observed at temperatures above 42°C and the morphology of  $\Delta pra1$  *C. albicans* was the same as wild-type at 37°C (24), the temperature at which we grew the  $\Delta pra1$  strain. We further observed that the morphology of the  $\Delta pra1$  and wild-type *C. albicans* used in our studies was similar. Hence, the inability of the  $\Delta pra1$  *C. albicans* to support leukocyte adhesion or migration is likely due to the absence of Pra1p rather than secondary to a change in morphology. This interpretation is supported by our studies using *PHR1*<sup>null</sup> mutant strains. The  $\Delta phr-1$  mutant germinates normally at pH 5.5 and 7.4 (32, 43) and we obtained morphologically normal fungal cells with hyphal structures indistinguishable from wild-type *C. albicans* as verified by microscopic examination. The  $\Delta phr-1$  cells were unable to support leukocyte adhesion and lacked Pra1p expression as confirmed by western blots, consistent with previous reports (23). In a complementation analysis, we showed that a “rescued” *Phr1* strain re-expressed Pra1p and reacquired the ability to support leukocyte adhesion and migration.

Our conclusion that Pra1p is an  $\alpha_M\beta_2$  ligand is consistent with several of its known properties. Pra1p, when associated with the fungal cell wall, is localized primarily or exclusively to the hyphal structures depending on the *Candida* strain (44, 50, 55) and leukocytes adhere only to the hyphal filaments, and not to yeast form of *Candida*. Furthermore, leukocytes use  $\alpha_M\beta_2$  to adhere to the hyphal structures (16).

Lopez-Ribot (56) isolated Fbp1p/Pra1p as a large multisubunit/multiprotein complex with molecular masses of >250–280 kDa and we isolated Pra1p within such a complex as well and identified two of the proteins within this complex as mannoprotein MP65 (37) and the 93.7-kDa hyphally regulated protein precursor (38). Whether these interactions occur on the fungal surface, in the releasate, or during concentration of the CAS are possibilities that cannot be distinguished at this time.

The physiological roles of Pra1p as a cell surface protein are still not definitively established. Pra1p is a fibrinogen-binding protein (36, 56, 57) and microorganisms use fibrinogen/fibrin as an adhesive substrate for colonization (58). Pra1p contains collagen-like sequences, which may also be involved in anchoring and colonization (59). Its regulation of hyphal formation may account for the role of Pra1p in *C. albicans* virulence (57, 60, 61). In our experiments, addition of soluble Pra1p to a system containing *C. albicans* and neutrophils increased fungus survival. It is known that soluble bacterial or fungal cell wall components are powerful Ags and complement activators, and one of the main immunological evasion strategies used by microbial pathogens is the ability to release surface ligands in a soluble form to neutralize host defense mechanisms. For example, soluble decoys are used by the Shope (62) and pox viruses (63) to evade host responses. Our data suggest that released Pra1p may play a pivotal role in the fungus defense arsenal: soluble Pra1p may compete with hyphal-anchored Pra1p for recognition by  $\alpha_M\beta_2$  and block leukocyte adhesion to *Candida*, an interaction which is critical in the killing of the fungus. Interestingly, MP65 was found in complex with Pra1p in CAS. MP65 has been implicated in immunomodulation of host defense by *C. albicans* and is a main target of human T cell responses to the fungus. Nanogram doses of purified MP65 are sufficient to induce extensive human T cell proliferation (37, 64). Thus, the release of a Pra1p/MP65 complex may function as prominent suppressor of the host defense system.

## Acknowledgment

We thank Dr. M. Kinter of the Lerner Research Institute of Cleveland Clinic for mass spectrometry analyses.

## Disclosures

The authors have no financial conflict of interest.

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