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Purification and Biochemical Characterization of a 65-Kilodalton Mannoprotein (MP65), a Main Target of Anti-*Candida* Cell-Mediated Immune Responses in Humans

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A 65 kDa-constituent (MP65) of a whole-cell mannoprotein (MP) fraction of *Candida albicans* was purified by immunoaffinity chromatography with monoclonal antibodies directed against periodate-insensitive, protease-sensitive MP epitopes, putatively polypeptide in nature. These antibodies were obtained by immunization of mice with concanavalin A bead-coupled, low-glycosylated MP from hyphal cells of *C. albicans* grown in the presence of a subinhibitory dose of tunicamycin. The immunoaffinity-purified MP65 molecule had a pI of 4.1 and a protein/polysaccharide ratio of 1.8:1. It was resistant to hydrolysis by endoglycosidase H, endoglycosidase F, or *N*-glycanases but still reactive with concanavalin A. The polysaccharide moiety of MP65 was composed exclusively of mannose and glucose at a ratio of 12.7 to 1. The protein moiety showed numerous potential O-glycosidic linkage sites as suggested by the high proportion of serine and threonine (together accounting for more than 20% of the total amino acid composition) and susceptibility to diluted alkali. This treatment and digestion with α-mannosidase caused a reduction in the MP65 molecular mass to around 54 kDa. The N-terminal sequence of MP65 protein moiety was rich in alanine and valine (7 of 13 amino acids) and did not show any significant homology with deposited sequences in data banks. Purified MP65, at doses of a few nanograms, induced extensive T-cell proliferation of human peripheral blood mononuclear cells. This proliferation was specifically inhibited, in a dose-response fashion, by the antigen-binding fragment of the monoclonal antibody used for immunoaffinity purification. Overall, these results highlight biochemical and molecular details of MP65, a main target of human T-cell response to *C. albicans*.

Candida albicans is an opportunistic fungal pathogen that has become an increasingly common cause of disease in the immunocompromised or otherwise modified host (22). Although this fungus possesses an array of putative virulence traits (17, 34), a defective immune response is usually a prerequisite for serious candidal infections. In particular, clinical and experimental evidence suggest that T cells are critical for anti-*Candida* immunosurveillance (10, 22, 26, 38–40). However, very little is known about the specific antigen targets of the T-cell response in humans and their protective nature.

In the context described above, we have long been studying the main mannoprotein (MP) antigens expressed by *C. albicans*, their immunogenicity, and their potential protective ability in both systemic and mucosal infections by the fungus (2–4, 12–14, 16, 30, 44–46). From an acidic MP material of a *Candida* whole-cell extract (MP-F2), we have recently prepared a 65-kDa MP (hereafter referred to as MP65)-rich fraction that induced extensive human T-cell proliferation in vitro. This proliferative response was of an antigenic rather than a mitogenic type and was likely targeted against epitopes of the protein moiety of the mannoprotein fraction (16, 44). A similar MP constituent was also abundantly released from *C. albicans* into the culture medium and was more active, on a weight basis, than the whole-cell MP preparation in inducing lymphoproliferation (7, 45). Despite several attempts for conventional purification of this major antigenic constituent of *C. albicans*, the molecular polydispersity and heterogeneity of the MP material eventually led only to fractions with increased MP65 content, which were unsuitable for further molecular characterization of the antigen.

In this investigation, methods were designed to produce monoclonal antibodies (MAbs) directed against putative nonmannan epitopes of the molecule which could allow immunoaffinity purification of the MP65 antigen. By this approach, a purified MP65 molecule was characterized biochemically and partially sequenced. This purified constituent proved to be a potent T-cell immunogen in cultures of human peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

Organisms and culture conditions. *C. albicans* BP, serotype A, from the established stock collection of the Istituto Superiore di Sanità, was used throughout this study. Its origin, culture maintenance, and growth conditions have been described elsewhere (7, 44, 45). For growth in the presence of tunicamycin (Sigma Chemical Co., St. Louis, Mo.), the MIC for this strain (2.5 μg/ml) was first determined by a microtiter assay, and a subinhibitory concentration of the drug (1 μg/ml) was used for large-scale cultures.

Antigenic preparations. The MP65-enriched preparation from whole yeast cells of *C. albicans* (F2-MP65) was obtained as reported previously (3, 46). Briefly, a crude MP extract obtained from the supernatant of autoclaved yeast cells by cold ethanol precipitation was subjected to DEAE-Sephadex A50 chromatography, and an acidic fraction (MP-F2) was eluted with 0.25 M NaCl. MP-F2 was subsequently separated by gel permeation in Sephacryl S300 HR, and homologous fractions with the highest content of MP65 were pooled to obtain the F2-MP65 preparation.

Mannoproteins spontaneously released from *C. albicans* cultures (sMP) were prepared in Lee's medium (28) buffered in 0.1 M phosphate buffer (pH 6.5) as described elsewhere (7, 45), with or without the addition of tunicamycin (see above). Starting cultures of 0.5 optical density (560 nm) were incubated at either 28 or 37°C for 24 h to obtain cells in the yeast or hyphal form, respectively. After checking the cell morphology by microscopy, the supernatants were collected by rapid filtration through a 0.8-μm-pore-size nitrocellulose membrane filter (Mil-

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lipore Corp., Bedford, Mass.). The sMP were concentrated and dialyzed in an ultrafiltration device equipped with a low-adsorbance membrane with a 10-kDa molecular mass cutoff (Diaflow Ultrafilter YM10; Amicon Corp., Danvers, Mass.). In some experiments, the sMP from hyphal cells (M-sMP) were further fractionated by gel filtration chromatography on Sephacryl S300 HR, as described previously (7), to obtain a fraction enriched in the MP65 component. Unless otherwise specified, the experiments used the M-sMP material described above as the starting source of the MP65 constituent.

Analytical determinations. Total polysaccharide content was measured by the phenol-sulfuric acid method (20), with mannose as the standard. The protein concentration was measured by the Bio-Rad (Hercules, Calif.) Dc protein assay, with bovine serum albumin (BSA) as the standard.

SDS-PAGE and Western blotting (immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (27) with a minigel system (mini-Protean II dual slab cell; Bio-Rad). Samples were separated in 5 to 15% polyacrylamide slab gels, with a 3.5% acrylamide stacking gel, at a constant voltage of 200 V for 50 min. After electrophoresis, MPs were either stained with the Bio-Rad silver stain kit or transferred electrophoretically onto nitrocellulose (0.2-μm pore size) or polyvinylidene difluoride (PVDF) membranes in a Trans Blot cell (Bio-Rad). Transfer was carried out at a constant voltage of 30V for 1 h, and the membranes were then reacted with either concanavalin A (ConA) or MAbs (see below).

For MP detection by ConA, nitrocellulose sheets were first blocked with 3% BSA in 0.1 M Tris-HCl [pH 7.5] containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 150 mM NaCl (BSA-TCM) for 1 h at room temperature and then incubated with ConA-digoxigenin (Boehringer GmbH, Mannheim, Germany) diluted 1:1,000 in BSA-TCM for 1 h at room temperature. After three washes with TCM, the sheets were incubated with phosphatase alkaline-conjugated antidigoxigenin (Boehringer) diluted 1:1,000 in BSA-TCM for 1 h at room temperature. Reactive bands were developed with nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolyl phosphate.

Immunoblot analyses were performed essentially as described previously (7, 8, 44, 45). Hybridoma supernatants and purified MAbs (1.5 mg/ml) were used at 1:4 and 1/1,000 dilutions, respectively, and detected with alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG, γ specific; Sigma) at the manufacturer's recommended dilution.

Isoelectric focusing. Isoelectric focusing was carried out with a Multiphor II electrophoresis unit (Pharmacia Biotech, Uppsala, Sweden) cooled with a thermostatic circulator. Electrode solutions were 1 M H_3PO_4 and 1 M NaOH. Samples were applied to precast 5% polyacrylamide gels (Ampholine PAG plate, pH 3.5 to 9.5; Pharmacia Biotech). After focusing, the gels were fixed in 30% methanol–10% trichloroacetic acid–3% sulfosalicylic acid for 1 h, the ampholytes were removed with several changes of 30% methanol–12% trichloroacetic acid for 2 h, and the gels were stained with a silver stain kit (Bio-Rad). Isoelectric point standards were as follows: trypsinogen, 9.30; lentil lectin, 8.65, 8.45, and 8.15; horse myoglobin, 7.35 and 6.85; human carbonic anhydrase B, 6.55 and 5.85; β-lactoglobulin A, 5.20; soybean trypsin inhibitor, 4.55; and amyloglucosidase, 3.5.

Enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates (Dynatech, PBI, Milan, Italy) were coated with the antigen (2 μg of polysaccharide per ml) in 50 mM carbonate buffer (pH 9.6) by overnight incubation at 4°C. The test was performed as described elsewhere (7, 45), with alkaline phosphatase-conjugated goat anti-mouse immunoglobulins and *p*-nitrophenyl phosphate disodium tablets (Sigma 104) as the substrate.

Dot blot. Dot blot immunoassay was performed with a Bio-Dot microfiltration apparatus (Bio-Rad). Twofold dilutions of the MP samples in 20 mM Tris-HCl (pH 7.5)–0.5 M NaCl were applied to nitrocellulose membranes (0.45-μm pore size; Bio-Rad) as described in the manufacturer's instructions. Filters were allowed to dry at 37°C and then processed for ConA or MAb immunodetection as described above.

Preparation of anti-MP65 MAbs. Eight- to 10-week-old female BALB/c mice (Charles River, Calco, Italy) were immunized by intraperitoneal injection of four doses of an MP65-enriched fraction from M-sMP material. For the first two doses, 75 μg of polysaccharide was coupled to 25 μl of ConA-agarose beads (12 mg of ConA per ml; Sigma) by overnight incubation in TCM at 4°C. Beads were brought to 0.75 ml with bidistilled H_2O and emulsified into an equal volume of complete Freund adjuvant. Two doses of this preparation were given to each mouse at a 3-week interval. Mice received a third dose of 25 μg of the soluble antigen in incomplete Freund adjuvant, and after 1 week, immunization was confirmed by ELISA (see above). A final booster of 50 μg of antigen in saline was given to the mouse showing the highest titer in the ELISA, 4 days before fusion.

Mouse spleen cells were fused, at a 4:1 ratio, with the myeloma cell line X63-Ag8.653, and hybrids were selected and maintained as reported elsewhere (15, 28). Supernatants of vigorously growing hybridomas were tested twice for specific antibody production by ELISA, with the immunizing antigen as the coating antigen and alkaline phosphatase goat anti-mouse polyvalent immunoglobulin (Sigma) as the conjugate. Positive hybridomas were subsequently expanded and tested for antigenic specificity by Western blotting. Antibody class and subclass were determined by ELISA with the specific alkaline phosphatase conjugates anti-IgM, -IgG1, -IgG2a, -IgG2b, and -IgG3 (Sigma). The selected hybridomas were subcloned twice by limiting dilution and subsequently grown in vivo in pristane-treated BALB/c mice. MAbs were purified from ascitic fluids by affinity chromatography on protein A-Sepharose 4 Fast Flow (Pharmacia). Two MAbs (7H6 and 4H12 of isotypes IgG2b and IgG2a and recognizing the MP65 constituent and an associated 70-kDa MP, respectively) were employed for immunoaffinity purification of MP65 as described below.

Other MAbs. Several MAbs raised against MPs of *C. albicans* were also used throughout this study. Our MAbs AF-1 (IgM) and 1D10 (IgG1), recognizing distinct MP polysaccharide epitopes present in the cell wall of *C. albicans* have been described elsewhere (15, 32). MAbs B9E and N3P (IgM) specific for different epitopes of serotype A mannan (37) were kindly given by J. Ponton, Universidad del Pais Vasco (Bilbao, Spain). MAb 11C11 (IgM), kindly furnished by M. Senet, University of Angers (Angers, France), binds to the fibrillar layer and to a 45- and a >200-kDa MP fraction (48); MAbs 5B2 and 1B12 (both IgM), reacting with mannan of *C. albicans* (46), were kindly provided by D. Poulain, Institut National de la Santé et de la Recherche Médicale, Lille, France.

Immunoaffinity chromatography. Affinity columns (1.6 by 2.5 cm) were prepared by covalently coupling the selected MAbs (4H12 or 7H6) to protein A-Sepharose CL-4B beads (Pharmacia) with dimethylpimelimidate as the cross-linker. The purified antibodies, resuspended in 0.1 M Tris-HCl (pH 8.0), were bound to protein A at a final concentration of 2 mg/ml of packed gel for 1 h at room temperature. After washing with 50 ml of 0.2 M sodium borate (pH 9.0), the beads were resuspended in 50 ml of the same buffer, and dimethylpimelimidate was added to a 20 mM final concentration. After 45 min of incubation, dimethylpimelimidate was discarded and the reaction was stopped by washing and incubating the beads with 50 ml of 0.2 M methanolamine (pH 8.0) for 2 h at room temperature. The gels were finally washed with 0.1 M Tris-HCl (pH 8), and the columns were prepared and stored in the same buffer.

For immunoaffinity purification of MP65, 4H12 and 7H6 columns were used in a series. Both columns were equilibrated with 10 mM Tris-HCl–0.5 M NaCl (pH 8.0), and the M-sMP material, dialyzed against the same buffer, was applied to the column at a flow rate of 1 ml/h. After washing with 50 ml of the same buffer, the two columns were disconnected and washed separately with 25 ml of 10 mM Tris-HCl (pH 8), followed by 25 ml of 10 mM Tris-HCl (pH 9). The bound material was finally eluted with 50 ml of 0.1 M triethylamine (pH 11.5). One-milliliter fractions were collected and neutralized with 1 M Tris-HCl (pH 6.7), and 1 μl of each fraction was tested in a dot blot for reactivity with MAb 7H6, MAb 4H12, and ConA. MAb 7H6-positive fractions were collected, concentrated, and dialyzed against double-distilled H_2O by ultrafiltration in Centriprep-10 and Centricon-10 devices (Amicon). The MP65 preparations thus purified were negative in the *Limulus* amebocyte lysate gelification test (Pyrotell; Biologi K-0, Trieste, Italy).

Chemical treatments. Periodate treatments were performed by incubating 100 μl of samples (100 μg of polysaccharide) with 100 μl of 0.1 M $NaIO_4$ in 0.2 M acetate buffer (pH 4.5) for 1 h at 4°C. The reaction was stopped with 200 μl of ethylene glycol. MP65 transblotted on PVDF membranes was also treated in the same way. For mild alkali treatment, samples (purified MP65 and M-sMP, 2 and 25 μg of polysaccharide, respectively) were resuspended in 0.1 M NaOH, incubated at 4°C for 2 h, and finally neutralized with 25 μl of 0.1 M HCl.

Enzyme treatments. Five micrograms of staphylococcal V8 protease (Sigma) was added to 20 μl of the purified MP65 (10 μg of protein) resuspended in 0.1 M Tris-HCl (pH 7.8). The mixture was incubated for 20 h at 37°C, and the digestion was stopped by heating at 100°C for 2 min in SDS-PAGE sample buffer. For pronase treatment, samples (100 μg of polysaccharide) resuspended in 100 μl of 0.1 M phosphate-buffered saline (PBS; pH 7.4) were incubated with 5 μg of pronase (type XXV; Sigma) overnight at 37°C. The enzyme was then precipitated by heating at 100°C for 10 min and discarded by centrifugation. Samples incubated under the same conditions, without the enzyme, were used as controls.

For treatment with endoglycosidases, samples from both MP65-enriched M-sMP (from cultures not treated with tunicamycin) or purified MP65 (0.5 mg of protein per ml) were denatured by boiling in 0.2% SDS–1% β-mercaptoethanol for 5 min. After cooling, 10 μl of each sample was treated with the following enzymes (Boehringer) diluted in 10 μl of the appropriate buffer: 2 to 20 mU of endoglycosidase H (endo H; EC 3.2.1.96) in 40 mM sodium phosphate buffer (pH 5.5), 100 to 500 mU of endoglycosidase F (endo F; EC 3.2.1.96) in 100 mM sodium acetate buffer (pH 6.0), and 600 mU of *N*-glycanase (glycopeptidase F or GNPase F; EC 3.2.2.18) in 100 mM sodium phosphate buffer (pH 7.5). Incubations were carried out at 37°C overnight in the presence of 0.5% Nonidet P-40–20 mM EDTA–1 mM phenylmethylsulfonyl fluoride–0.02% sodium azide. Similar aliquots of purified MP65 were also treated with 20 to 100 mU of jack bean α-mannosidase (Boehringer) in 150 mM sodium acetate buffer (pH 4.5), under the same conditions as those described above. Before incubation with our MP preparations, the genuine activity of all the enzymes described above was controlled by the use of appropriate glycoprotein substrates. Moreover, samples incubated under the same conditions but without the enzyme were used as controls.

N-terminal amino acid sequencing. Affinity-purified MP65 (10 μg) was subjected to SDS-PAGE under reducing conditions and transblotted onto PVDF membranes as described above. After three washes with bidistilled H_2O, membranes were equilibrated in 0.1 M acetate buffer (pH 4.5) and treated with a solution of 50 mM $NaIO_4$ in the same buffer. After 1 h of incubation, the periodate solution was discarded and the blots were washed extensively with bidistilled H_2O. The membrane was stained with 0.1% Coomassie blue R-250 in

50% methanol to visualize the MP65 band, which was then excised and subjected to automated Edman degradation in a gas-phase sequencer (model 476A; Applied Biosystems, Foster City, Calif.) with an optimized program (30).

Amino acid composition. Amino acid analysis was performed on both soluble, affinity-purified MP65 and on a PVDF-immobilized MP65 band. The immobilized band was prepared as follows: 16 μg (protein) of affinity-purified MP65 was subjected to SDS-PAGE, electrophoretically transferred at 30 mA for 75 min on a PVDF membrane, and stained with amido black. After 1 h of extensive washing with bidistilled H_2O, the stained MP65 band was excised, hydrolyzed in the vapor phase with 6 M HCl at 110°C for 24 h, and then rinsed three times with 100 μl of 0.1 N HCl in 30% methanol. The solutions were combined and dried under vacuum. A piece similar in size to that of the MP65 band was cut from an empty area of the PVDF membrane, treated in the same way, and used as a control. An aliquot corresponding to 60% of the total sample was subjected to amino acid analysis, which was performed by ion-exchange chromatography with a Beckman System Gold high-performance liquid chromatograph (HPLC; Beckman Instruments, Fullerton, Calif.) with postcolumn ninhydrin derivatization. The soluble MP65 (2 μg of protein) was hydrolyzed and analyzed as described above.

Sugar composition. The saccharide moiety of the immunoaffinity-purified MP65 was analyzed by HPLC. Samples were hydrolyzed with 2 N trifluoroacetic acid at 100°C for 5 h, and then aliquots corresponding to about 2 μg of saccharide were analyzed with an HPLC apparatus composed of a gradient pump module (GPM II; Dionex, Sunnyvale, Calif.) and a pulsed amperometric detector (Dionex). Sample injections were via a Dionex autosampler equipped with a 25-μl sample loop. Sugars were separated on a CarboPac PA1 column (250 by 4 mm), serially connected with a CarboPac PA guard column (25 by 3 mm), at a flow rate of 0.8 ml min^{-1}. Isocratic analysis with 16 mM NaOH as the eluant was performed for the separation of neutral and amino monosaccharides. Detection was by a pulsed amperometric detector with a gold working electrode. The following pulse potentials and durations were employed for detection: E_1 = 0.1 V (t_1 = 480 ms); E_2 = 0.6 V (t_2 = 120 ms); E_3 = −0.8 V (t_3 = 60 ms). All steps of the procedure described above were validated by the analysis of reference sugars.

Papain digestion. Fab fragments from MAb 7H6 were obtained by digestion of 10 mg of antibodies with 10 U of papain-agarose beads (Sigma) in a 1-ml final volume. Papain was activated in 1 ml of 20 mM phosphate buffer (pH 7.5)–20 mM EDTA–20 mM cysteine for 15 min at 37°C and then mixed with 1 ml of antibodies in 20 mM phosphate buffer (pH 7.5) for 6 h at 37°C. Fab fragments were purified by chromatography on protein A-Sepharose CL-4B, using the MAPS buffer (Bio-Rad) to bind Fc fragments and undigested immunoglobulin. Unbound Fab fragments were dialyzed against Dulbecco's PBS (Gibco). Their purity was assessed by SDS-PAGE and Coomassie blue stain, showing complete digestion, with the expected molecular size of the fragments, in both mercaptoethanol-containing and mercaptoethanol-free solutions. Moreover, they retained specific reactivity with MP65 as shown by their ability to compete with MAb 7H6 in binding to MP65 in a competitive inhibition ELISA (data not shown).

Lymphocyte proliferation assay. PBMC were isolated on density gradient (Lymphoprep; Nyegaard, Oslo, Norway) from venous blood samples of healthy human donors. The cells were washed in RPMI 1640 medium (Gibco, Grand Island, N.Y.), resuspended at 10^6 cells per ml in the same medium supplemented with heat-inactivated 5% pooled human AB serum, and cultured in 96-microwell trays (200 μl per well) in the presence of various doses of the stimulant indicated in each single experiment. All stimulants were endotoxin-free, as assessed by the *Limulus* amebocyte lysate gelification test. Human AB sera selected for the study were those batches containing the lowest amount of anti-*Candida* antibodies (i.e., undetectable by agglutination test) and a titer not higher than 1:100 by ELISA (with MP-F2 as the coating antigen). Cultures were incubated at 37°C under a 5% humidified CO_2 atmosphere. Cell proliferation was measured on day 7 of the culture by [^{3}H]thymidine incorporation, as reported previously (2–4). Assays were performed in triplicate, and each material was tested with at least three blood samples obtained from separate donors. In some experiments, Fab fragments from papain-digested MAb 7H6 (see above) were added to the 96-microwell trays containing the different stimulants used. After 18 h of incubation at 4°C, PBMC cultures were added to the plates and the lymphoproliferation test was performed as described above. Purified protein derivative (PPD) from *Mycobacterium tuberculosis* and human recombinant interleukin-2 (IL-2) were generous gifts of C. M. Ausiello (Istituto Tipizzazione Tissutale, Consiglio Nazionale delle Ricerche, Rome, Italy). They were used as control stimulants of PBMC cultures, as described elsewhere (4).

RESULTS

Biochemical and immunogenic characteristics of the sMP from tunicamycin-treated yeast and mycelial cells of *C. albicans*. MP65-enriched fractions from sMP were, like those obtained from the previously described MP-F2 cell fraction of *C. albicans* (2, 46), poorly immunogenic in mice, since low numbers of MAb-secreting hybridomas with broad specificity for the mannan moiety of MP were obtained in all cases (preliminary experiments; data not shown). To increase the likelihood of obtaining MP65-specific MAbs for immunoaffinity purification of the antigen, a low-glycosylated, protein-enriched MP from tunicamycin-grown hyphal cells (M-sMP), coupled to ConA beads, was used as an immunogen. Chemical determinations of five different batches of this preparation showed an average polysaccharide/protein ratio of 1:2, a marked reduction from the 3:1 ratio of the same components in the correspondent material from tunicamycin-untreated cells.

TABLE 1. Proliferation of human PBMC stimulated by mycelial secretory materials (M-sMP) from untreated or tunicamycin-treated *C. albicans* cultures

Material	Polysaccharide/ protein ratio	Dose[a] (μg/ml)	Lymphoproliferation[b]	
			Donor 1	Donor 2
None			0.5 ± 0.1	0.5 ± 0.2
MP-F2	9:1	50	17.6 ± 1.2	30.1 ± 2.6
M-sMP	3:1	1	22.0 ± 1.7	35.7 ± 1.5
		0.1	17.1 ± 0.8	22.1 ± 0.9
M-sMP-tunicamycin[c]	0.5:1	1	23.8 ± 1.4	33.0 ± 3.9
		0.1	22.2 ± 0.1	22.4 ± 3.4

[a] Expressed as micrograms of polysaccharide per milliliter of PBMC culture.
[b] Evaluated as [^{3}H]thymidine incorporation and expressed as counts per minute (10^3) per 10^6 cells (mean ± standard deviation).
[c] Mycelial secretory mannoproteic material released from tunicamycin-grown cells of *C. albicans.*

To validate our approach, an obvious prerequisite was that protein-enriched M-sMP had preserved its biological activity. Table 1 shows that M-sMP were indeed very effective in the induction of proliferation in human PBMC cultures from two *Candida*-responsive healthy donors. In particular, the lymphoproliferative response achievable with 0.1 μg of the low-glycosylated antigenic secretion from tunicamycin-grown hyphal cells was comparable to that resulting from stimulation by 50 μg of MP-F2 fraction (46), showing that lymphoproliferation-inducing ability was paralleled by progressive enrichment for protein in the mixture.

MAbs against the MP65 constituent of the mycelial secretion. Mouse immunization with ConA-bound M-sMP from tunicamycin-grown *C. albicans* cells gave high, specific antibody titers. After fusion, more than two different growing clones were seen in all seeded wells. On first screening with MP65-enriched material as the coating antigen, 224 of 960 tested wells were positive, and after a second screening, the hybridoma cultures from 120 positive wells were selected and expanded. Most of them (92%) secreted immunoglobulins belonging to the IgG class.

To identify MAbs reacting with nonmannan, putative protein epitopes, the supernatants of all of the expanded cultures were first analyzed by dot blotting with either pronase- or periodate-treated M-sMP, and then the specificity of the antibodies was examined by Western blotting. The clone 7H6 (an IgG2b-secreting culture) was selected for its strong reactivity with MP65, and the MAb was purified from ascites for subsequent studies. Another fusion product (MAb 4H12, an IgG2a) was also selected and used for affinity purification of MP65 (see below).

Dot blot reactions between MAb 7H6 and yeast or mycelial sMP as well as with a crude preparation of MP65 from the MP-F2 fraction of *C. albicans* (44, 46) showed that all of these materials were reactive with the antibody. The reaction was, in all cases, more intense with periodate-treated antigens and negative with protease-treated antigens. In contrast (and as a control), periodate but not protease treatment abolished the reactions between the same antigenic materials and both

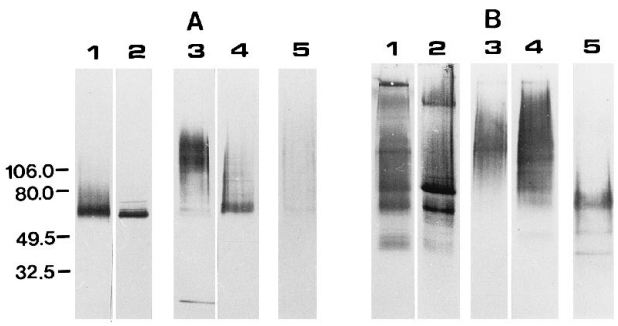

FIG. 1. Western blot analysis of MP65 from mycelial (lanes 1 and 2) or yeast (lanes 3 and 4) secretory materials and MP65-enriched MP-F2 (lane 5). Secreted MPs were obtained from cultures grown in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of tunicamycin. (A) Reaction with MAb 7H6; (B) ConA stain. The loaded amounts were (in polysaccharide) 5 μg for the mycelial secretion, 20 μg for the yeast secretion, and 40 μg for MP-F2. Molecular mass markers are in kilodaltons.

ConA (which reacts with the α-mannan moiety of MP) and MAb AF1, which recognizes β(1-2)-linked oligomannoside chains, also present in *C. albicans* MP (15, 47). This reactivity pattern was exactly the same when either the yeast or mycelial sMP from tunicamycin-treated cells was used (data not shown).

The specificity of dot blot reactions was confirmed by Western blotting (Fig. 1), which showed that MAb 7H6 was directed against a 65-kDa band. This band was essentially the only reactive constituent of M-sMP (Fig. 1A, lane 1), the polydispersity of which was substantially abolished by tunicamycin treatment (Fig. 1A, lane 2). In the secretion material of the yeast culture (Y-sMP), the MAb 7H6 epitope was contained in a highly polydisperse, >100-kDa material (Fig. 1A, lane 3). Growth in the presence of tunicamycin brought about a substantial loss of this polydispersity, and again, the MP65 band was a major reactive constituent (Fig. 1A, lane 4). MAb 7H6 also reacted with a polydisperse MP of the MP65-enriched MP-F2 fraction (Fig. 1A, lane 5), but the amount of the antigen present in this fraction was very low, as judged from the intensity of the stain and the amount of polysaccharide (40 μg) that was necessary for reaction.

The specificity of MAb 7H6 reactions was confirmed by the comparison with the ConA blots of the same materials (Fig. 1B). As expected, these blots showed the presence in both Y- and M-sMP of an elevated number of mannoproteic constituents of various molecular weights, among which a reactive MP65 band also predominated in the secretion of tunicamycin-treated mycelial cultures.

Immunoaffinity purification of MP65. MAbs 4H12 and 7H6 were used in sequence for immunoaffinity purification of MP65 antigen after antibody coupling to protein A-Sepharose (see Materials and Methods). Since MP65 copurified with a 70-kDa MP when only an immunoaffinity column with MAb 7H6 was used (data not shown), an affinity precolumn was used with an MAb (4H12). This column was able to bind both the 70-kDa MP and other nonspecific MP material, while the column with MAb 7H6 retained full MP65 binding capacity. In these and all subsequent experiments, the source of the MP65 constituent was the M-sMP from tunicamycin-treated cells. As shown in Fig. 2, the procedure adopted allowed a consistent MP65 purification, as assessed by Western blots of the eluted material with ConA (Fig. 2A) and MAb 7H6 (Fig. 2B). The reaction with ConA was particularly homogeneous, without significant polydispersity, demonstrating that the affinity-purified MP65 was substantially free from other MPs (Fig. 2A, compare lanes 1 to 3; see also Fig. 1). The purity of different batches of this preparation was also assessed by silver stain (Fig. 2C). In all cases, the batches of purified MP65 have identical properties, and a single electrophoretic band of the expected molecular size was observed.

TABLE 2. Amino acid composition of SDS-PAGE-separated, PVDF-immobilized MP65 from *C. albicans*[a]

Amino acid residue	% Of total amino acids
Asp/Asn	9.8
Thr[b]	6.8
Ser[b]	15.1
Glu/Gln	3.9
Pro	3.9
Gly	13.6
Ala	12.0
Cys	ND[c]
Val	11.3
Met	0.0
Ile	5.2
Leu	3.9
Tyr	3.2
Phe	2.3
His	Traces
Lys	4.5
Arg	4.5
Trp	ND

[a] Values are expressed as percentages of each amino acid in the total, with the exception of Cys and Trp, which were not determined. The absolute amounts of each amino acid in the whole molecule could not be determined since the molecular weight of the protein moiety remained unknown.

[b] No correction was made for the destructive loss of Ser and Thr during hydrolysis.

[c] ND, not determined.

Biochemical and immunochemical characterization of purified MP65. Biochemical and immunochemical properties of immunoaffinity-purified MP65 constituent were further investigated. The molecule contained 1.8 parts of protein for 1 part of polysaccharide, as an average of three independent determinations on three different batches. The amino acid composition of the protein moiety was determined from two independent preparations of soluble or membrane-immobilized MP65, which gave overlapping results. As shown in Table 2

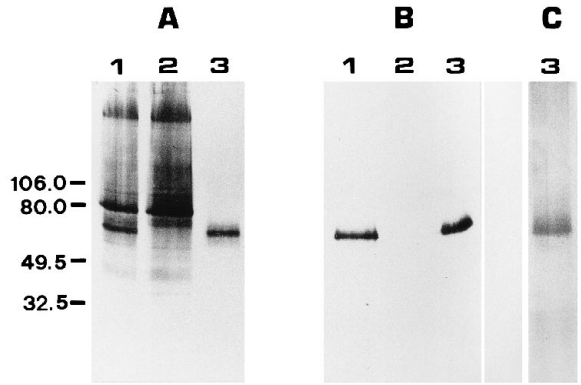

FIG. 2. Immunoaffinity purification of MP65. (A) ConA stain; (B) immunoreactivity with MAb 7H6; (C) silver stain. Lanes: 1, M-sMP from tunicamycin-treated cultures (4 μg of polysaccharide) as starting material; 2, the column-unbound material; and 3, the material eluted with 0.1 M triethylamine (50 ng). Molecular mass markers are expressed in kilodaltons.

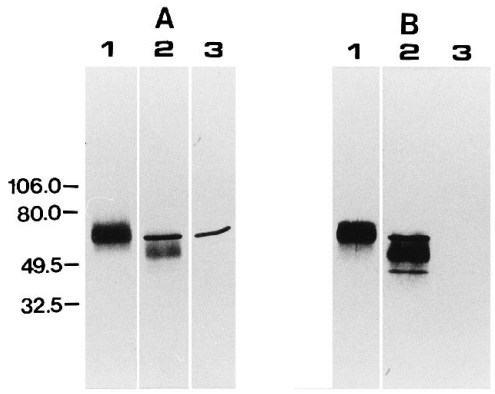

FIG. 3. Effects of α-mannosidase treatment on the purified MP65 as demonstrated by immunoblots with ConA (A) and MAb 7H6 (B). Lanes: 1, untreated sample (0.1 μg of polysaccharide); 2, the same amount of the sample after α-mannosidase treatment; 3, α-mannosidase only (control). The enzymatic preparation used contained numerous proteins as visualized by silver stain (data not shown). Two of these proteins, the major ConA-positive 65-kDa band (see panel A, lane 3) and another band of about 40 kDa, reacted with MAb 7H6 only after MP65 digestion (compare lanes 2 and 3) probably as a result of strong interactions with deglycosylation products containing MAb 7H6-reactive epitopes. Molecular mass markers are expressed in kilodaltons.

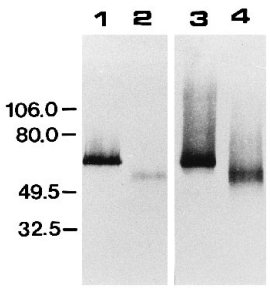

FIG. 4. Effect of mild alkali treatment on MP65, as shown by Western blot reactions of purified MP65 (lanes 1 and 2, untreated and NaOH-treated samples, respectively) and M-sMP from tunicamycin-treated cultures (lanes 3 and 4, untreated and treated samples, respectively) with MAb 7H6. Amounts loaded were 50 ng and 5 μg of purified MP65 and M-sMP, respectively. Molecular mass markers are expressed in kilodaltons.

(which reports the determination of the membrane-immobilized preparation), the protein was rich in acidic and neutral amino acids. Accordingly, the pI of the molecule fell in the acidic range (around 4.1), although the contribution of negative charges possibly imparted to the molecule by phosphodiester-linked oligomannoside chains cannot be ruled out (33, 42). Potential O-glycosidic linkage sites (in particular, serine) were numerous, together accounting for more than 20% of the total amino acid composition.

The N-terminal sequence of the protein, after periodate oxidation, was determined to be Ala-Val-His-Val-Val-Arg-Ser-Glu-Ala-Tyr-Ala-Gln-Val/Ser. This sequence does not show significant homology to protein sequences in the EMBL database. The purified MP65 was also treated with staphylococcal V8 protease, but this treatment generated inconsistent, ill-defined fragments, unsuitable for amino acid sequencing.

A strong reaction with ConA demonstrated that the purified MP65 constituent from tunicamycin-grown cells was still glycosylated. Chemical determinations and HPLC analysis showed that the saccharide moiety of MP65 contained almost exclusively mannose and glucose, but the former sugar was largely predominant (92.7% versus 7.3% of glucose, as an average determination of three samples). Only traces of *N*-acetylglucosamine were present. Although MP65 was not susceptible to deglycosylation by endo H, endo F, or *N*-glycanases, it was susceptible to α-mannosidase treatments (Fig. 3) as well as diluted alkali, known to hydrolyze O-labile (β-eliminable) side chains of α-mannan (33) (Fig. 4). Treatment with α-mannosidase did not affect the intensity of the MAb 7H6-reactive MP65 band, but it decreased the intensity of the ConA reaction (Fig. 3). Both treatments with mannosidase and with alkali reduced the apparent molecular mass of MP65 to around 54 kDa. Finally, none of several MAbs known to be directed against various, largely shared oligomannoside epitopes of MP (32, 37, 47) reacted with the purified MP65, suggesting that the polysaccharide moiety of this constituent has a rather specific structure.

Immunological activity of purified MP65. The purified and chemically characterized MP65 constituent of M-sMP from tunicamycin-treated cells of *C. albicans* described above was used in lymphoproliferation assay in cultures of lymphomonocytes from the peripheral blood of *Candida*-responsive healthy human donors. In the same experiments, the Fab fragments of the MAb 7H6 were used as potential inhibitors of the MP65 recognition, while PPD or IL-2 was used as an antigenic or polyclonal stimulator, respectively. The experiments were repeated five times with different donors, and comparable results were always obtained. Table 3 shows the results of one experiment with three responsive donors. The purified MP65 was a particularly potent inducer of lymphoproliferation, with high thymidine incorporation values even at a concentration of 10 ng/ml of culture. The proliferation index was comparable to, and even greater than, that obtained at the usual dose of 50 μg of MP-F2 per ml, i.e., the material from which a crude preparation of MP65 was first obtained (44, 46).

The addition of suitable amounts of MAb 7H6 Fab significantly reduced the proliferation induced by the MP65 constituent but not those stimulated by a *Candida*-unrelated antigen such as PPD or IL-2. Actually, the 7H6 Fab caused a marked

TABLE 3. MP65-induced lymphoproliferation in human PBMC cultures and its inhibition by MAb 7H6 Fab

Stimulator (concn)	7H6 Fab concn (μg/ml)	Cell proliferation[a]		
		Donor 1	Donor 2	Donor 3
None		1.1 ± 0.3	0.9 ± 0.3	0.9 ± 0.4
	5	1.2 ± 0.7	2.9 ± 0.2	2.6 ± 0.1
	50	0.5 ± 0.1	2.6 ± 0.3	2.8 ± 0.1
MP65 (0.1 μg/ml)		25.8 ± 2.4	44.1 ± 3.4	45.0 ± 3.3
	5	30.0 ± 1.9	45.0 ± 6.6	31.5 ± 3.2
	50	14.9 ± 2.1[b]	24.2 ± 1.5[b]	21.9 ± 2.1[b]
MP65 (0.01 μg/ml)		19.5 ± 2.2	49.1 ± 2.2	41.3 ± 3.8
	5	21.9 ± 4.5	27.3 ± 0.1[b]	23.1 ± 2.5[b]
	50	5.9 ± 0.7[b]	8.7 ± 4.3[b]	17.6 ± 0.5[b]
MP-F2 (50 μg/ml)			30.1 ± 2.6	34.7 ± 2.4
	5		12.0 ± 0.3[b]	17.7 ± 1.1[b]
	50		9.4 ± 2.4[b]	5.8 ± 0.4[b]
PPD (10 μg/ml)		56.3 ± 5.9	26.1 ± 2.0	54.9 ± 1.7
	5	53.0 ± 6.2	28.1 ± 0.3	70.6 ± 3.1
	50	48.6 ± 5.6	21.1 ± 0.5	54.5 ± 3.1
IL-2 (100 U/ml)		36.2 ± 4.2	36.6 ± 2.2	ND
	5	131.4 ± 16.9	80.5 ± 13.6	ND
	50	106.1 ± 3.9	70.1 ± 10.4	ND

[a] Evaluated as [^{3}H]thymidine incorporation and expressed as counts per minute (10^3) per 10^6 cells (mean ± standard deviation).

[b] $P < 0.05$ (Student's t test) compared with incorporation values of the respective PBMC cultures with no Fab addition.

increase of IL-2- or PPD-stimulated PBMC proliferation. Importantly, MAb 7H6 Fab fragments were also able to significantly reduce the MP-F2-induced lymphoproliferation, further confirming the initial assumption that MP65 was at least one of the active antigenic stimulators present in the MP-F2 fraction.

DISCUSSION

In this investigation, a procedure was devised to obtain MAbs potentially directed against nonmannan, putative protein epitopes of *C. albicans* MP. Two antibodies (MAbs 7H6 and 4H12) obtained by this procedure were used for immunoaffinity purification of an immunodominant antigenic constituent of *C. albicans* (MP65), and the Fab portion of MP65-specific MAb 7H6 was used to demonstrate the specificity of the antigenic recognition of MP65 epitopes by human PBMC proliferation assays in vitro. A number of biochemical and molecular characteristics of MP65 were therefore elucidated.

MPs of *C. albicans* and other fungi contain dominant B-cell mannan epitopes to which an antibody response is made strong and probably T cell dependent by natural conjugation with protein (18, 19, 21, 33, 35, 36). Thus, immunization with native MP usually results in a largely predominant production of antimannan antibodies, prevalently of the IgM isotype, which are unsuitable for immunoaffinity purification of molecules sharing largely similar oligomannoside epitopes. Other more conventional means (ion-exchange chromatography, gel permeation, and reverse-phase HPLC) are usually incapable of separating, with a reasonable degree of purity, such highly polydisperse, highly glycosylated protein molecules (33).

The procedure devised here to obtain MAbs suitable for MP immunoaffinity purification has two critical steps. The first is the use of tunicamycin, a well-known inhibitor of N-glycosylation, resulting in an MP-containing secretion mixture with fewer N-linked mannan chains and thus an enhanced relative proportion of protein in the mixture. In addition, the secretion mixture from hyphal rather than yeast cells of *C. albicans* was employed as an MP65 source, in view of the previous observations that the M-sMP were intrinsically less glycosylated than the Y-sMP, with more abundant constituents of low molecular mass (<100 kDa), inclusive of MP65 itself (7, 45). As documented elsewhere (7, 44), the M-sMP were more immunogenic than the Y-sMP, a property that was attributed to the greater amount of MP65 antigen in M-sMP than in Y-sMP. These previous findings have been confirmed and extended by the current observation that M-sMP from tunicamycin-treated cells, with an even lower degree of MP polydispersity and an increased protein/polysaccharide ratio (in comparison with the M-sMP from untreated mycelial cultures), retained a potent immunogenic potential.

The second relevant step of our approach to obtain MAbs suitable for MP65 purification was the immunization with ConA-bound M-sMP material. We hypothesized that coating with ConA could probably mask most of the B-cell mannan epitopes, leaving accessible for immune recognition at least some of the nonmannan, putative protein epitopes. Other factors, however, could have played a role in the high yield of MAbs directed against nonmannan epitopes of MP. For instance, ConA is a well-known polyclonal T-cell activator in mice but also exerts inhibitory effects on antibody production against polysaccharide antigens (49). Moreover, a nonneglectable positive effect could have been played by the ConA beads themselves, which render the whole MP-ConA complex a particulate one, possibly endowed with strong adjuvanticity.

The definition of the epitope recognized by the MAb 7H6 as a nonmannan, putative protein should be regarded as being purely operational, since it relies upon such indirect criteria as epitope susceptibility to protease digestion and resistance to periodate oxidation. Because this latter treatment does not affect β1-3 glucan, and the MP65 polysaccharide moiety contains some glucose, our MAb could theoretically be directed against a β1-3 glucan. It is unlikely, however, that any β-saccharide epitope is so intensely affected by protease digestion to result in a total loss of reactivity against MAb 7H6 while the same proteolytic treatment has no effect on the reactivity of other MAbs directed against β-saccharide configuration. It seems also unlikely that inhibition of lymphoproliferation by the 7H6 Fab (see below) could take place through binding to β1-3-glucan. It has been shown that protein is essential for MP-induced lymphoproliferation, which is of an antigenic, not a mitogenic, type, thus requiring protein processing by antigen-presenting cells (2, 3, 14, 44, 46). Clearly, the identification of the true chemical nature of the MP65 epitope recognized by the MAb 7H6 must await a more refined molecular characterization of the antigen.

Although MP65 contained an elevated proportion of mannan, as judged by the elevated mannose content and strong ConA reactivity, it was not recognized by a number of MAbs against common α- or β-mannan epitopes on a large variety of MP molecules (32, 37, 47, 48). Moreover, it was resistant to digestion by endoglycosidases which usually remove at least part of the mannan moiety of other MPs. The particular procedure by which MP65 was prepared, inclusive of growth in partially N-glycosylation-blocked cultures by tunicamycin, certainly provides fewer sites for endoglycosidase digestion. However, this cannot be the only reason for the absolute resistance to all of the enzymes described above since the MP65 constituent from other tunicamycin-untreated MP preparations (7, 44) was also substantially resistant to endoglycosidases (data not shown).

The only two treatments which showed some effect on molecular mass of MP65 were digestion with α-mannosidase and β-elimination with diluted alkali. Both treatments reduced the MP65 molecular mass by about 15%, suggesting that they could have affected the same O-linked oligomannosides. An indirect clue that the MP65 is extensively O-glycosylated is derived from the elevated proportion of serine and threonine, which bring O-linked oligosaccharide chains in MP (33, 42), together accounting for more than 20% of the whole amino acid composition.

There are several *C. albicans* glycoprotein antigens which approximate the molecular weight of the MP65 constituent, inclusive of receptors for soluble host factors of immunity and components of the so-called multifunctional adhesin of *C. albicans* (5, 6, 9, 11, 24, 25, 48, 50). They are cell surface located, and some of them have been shown to be immunogenic. For instance, the C3d-binding protein (a doublet of 55 to 60 kDa and with a pI of 3.9 to 4.1) (41) was antigenically expressed and induced T-cell proliferation in splenocyte cultures from *C. albicans*-infected mice (23). Comparisons are made difficult by lack of a biochemical or molecular characterization of most of the constituents described above. In particular, no N-terminal amino acid sequence similar to the MP65 sequence has been found in databases. Also, the amino acid composition of the 65-kDa fimbrial adhesin of *C. albicans* described by Yu et al. (50) differs substantially from that of MP65, and the same is true for the extracellular C3d-binding protein, as reported by Saxena and Calderone (41). This latter protein was also shown to be susceptible to endo F glycosidase, which was inactive on MP65, and to contain both mannose and *N*-acetylglucosamine but no glucose that is present in the MP65 purified preparation. In conclusion, speculations about the identity and physiological

functions of MP65 must await a complete characterization of both its protein and saccharide moieties and, possibly, the molecular cloning of the encoding gene(s).

The purified MP65 turned out to be a markedly more potent immunogenic molecule than a previous MP65-enriched preparation. While this latter preparation was active in inducing lymphoproliferation at doses ranging from 10 to 50 μg/ml (44), the purified MP65 induced an extensive lymphoproliferation at doses of a few nanograms, in the same assay and with the same PBMC donors. This is exactly what is expected on the basis of the assumption that MP65 is the active antigenic constituent of the MP preparations, as suggested previously (7, 44). Moreover, we have here obtained the first formal evidence that an epitope of MP65 itself, and not an unknown contaminant, is recognized by human T cells, as demonstrated by the strong and specific inhibition of MP65-induced lymphoproliferation in the presence of the Fab constituent of the MAb 7H6 used for MP65 purification.

Adaptive immune responses, particularly those mediated by T cells, play a fundamental role in the anti-*Candida* defense, as demonstrated by recurrent *Candida* infections in AIDS patients, in particular, those with low $CD4^+$ lymphocyte counts (26). Despite increased awareness of the importance of studying *Candida* antigens recognized by T cells to understand specific immune mechanisms underlying the control of candidiasis, very few of them have been identified to date (1, 43, 44). MP65 is, to our knowledge, the first immunodominant mannoprotein of *C. albicans* with a partially known amino acid sequence and a high degree of purity relative to this kind of molecule. The immunogenicity data obtained first with MP-F2 (2, 3, 46), then with MP65-enriched fractions from both intact cells and spontaneously released materials (7, 49), and now with immunoaffinity-purified MP65 leave little doubt that MP65 is indeed a major T-cell immunogen of *C. albicans*. In fact, we have previously shown that MP65- as well as MP-F2-induced human PBMC proliferation is inhibited by both anti-CD3–T-cell receptor and anti-major histocompatibility complex class II antibodies, requires antigen-presenting cells, and produces a typical T-helper type 1 cytokine pattern (2–4, 14, 31, 44). Since many other mannoproteic fractions from this fungus did not induce lymphoproliferation in vitro (3, 7, 29, 44, 45), MP65 is possibly the antigenic stimulator of other cell-mediated immune responses detected by use of uncharacterized mannoproteic extracts of the fungus (3, 33, 44, 45). Its elevated potency in inducing proliferation of T cells from all healthy subjects studied to date demonstrate that MP65 is expressed at a high level, in vivo, during the human commensal life of *C. albicans*. These data make the MP65 antigen a particularly suitable reagent to study the immune response to and protection against *C. albicans* in humans.

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