

Isolation and Characterization of a Major Antigenic Component of *Malassezia globosa* to IgE Antibodies in Sera of Patients with Atopic Dermatitis

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Abstract: Three major components of *Malassezia globosa* were isolated from 2-ME extracts of this fungus by ion-exchange column chromatography and are referred to as Malg46a, Malg46b and Malg67, respectively. IgE antibodies to these components in the sera of patients with AD were detected by immunoblots. In Western blot, IgE antibodies to Malg46b were most frequently detected in the sera of AD patients. Dot blot with the Malg46b-containing fraction immunologically reacted with 69% of the sera of the patients, and with 83% of the sera of the patients who were positive for IgE antibodies to the 2-ME extract of *M. globosa* in the Western blot. The intensities generated for each dot correlated well with the total intensities generated for the 2-ME extract of *M. globosa* in the Western blot ($r=0.763$). In the lectin blot, Con A reacted with both Malg46a and Malg46b but not with Malg67. The polyclonal antibody to Malg46b reacted strongly only with the 2-ME extract of *M. globosa* and reacted slightly with *M. restricta*. In conclusion, a glycoprotein, Malg46b of *M. globosa*, is dominantly expressed in this fungus and is a possible major antigen for IgE antibodies in patients with AD.

Key words: *Malassezia globosa*, Atopic dermatitis, IgE

The lipophilic fungus *Malassezia furfur* (also called *Pityrosporum orbiculare* and *P. ovale*) is often isolated as a common commensal from the surface of healthy human skin (11) or patients with atopic dermatitis (1). Many investigators have focused on the involvement of *M. furfur* in AD because immunoglobulin E antibodies specific for *M. furfur* were frequently found in patients with AD (14, 18), and several antigenic components of this fungus have been reported (4, 7, 8, 12, 15).

More recently, the genus *Malassezia* was classified into 7 species, *M. sympodialis*, *M. globosa*, *M. restricta*, *M. slooffiae*, *M. furfur*, *M. obtusa* and *M. pachydermatis*, by their morphological, biochemical and genetic characteristics (2, 16). However, the association of the new *Malassezia* species with AD and their major antigenic components which react with IgE antibodies in the sera

of AD have not been examined.

We have isolated *Malassezia* from patients and identified their species based on new classification methods (3, 13). In our previous study, IgE antibodies to *M. globosa* were often found in patients with AD and the IgE antibodies reacted frequently with ca. 46 kDa or ca. 67 kDa bands in Western blotting (manuscript submitted for publication). This suggests that these components are possible major antigens for IgE antibodies in patients with AD. We believe that defining the major antigenic components for the IgE antibodies of *M. globosa* will give us information useful not only for understanding the mechanism of AD but also for diagnostic standardization. The goal of our work is to define the major epitopes of the major allergens of *Malassezia* species for IgE antibodies in patients with AD. In this paper, we isolated a possible antigenic component for IgE antibodies from *M. globosa* and describe its character-

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; IgE, immunoglobulin E; RAST, radioallergosorbent test; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

istics.

Materials and Methods

Patients' sera. Sera were collected from 64 patients with atopic dermatitis (AD) who were diagnosed based on the Japanese Dermatological Association Criteria for the diagnosis of atopic dermatitis (17). For the control study, sera from 8 patients with seborrheic dermatitis (SD), 4 patients with chronic urticaria (CU), 2 patients with tinea versicolor (TV) and 10 healthy persons (HP) were used in this study. No patients had received systemic antifungal or corticosteroid treatments. All sera were stored at -80°C before use.

Three strains of *M. sympodialis* (NUM 6004, NUM 6013, NUM 6019), 3 strains of *M. globosa* (NUM 6006, NUM 6061, NUM 6062), 3 strains of *M. furfur* (NUM 6014, NUM 6030, NUM 6074), 2 strains of *M. restricta* (NUM 6005, NUM 6071) and *M. slooffiae* NUM 6003 were used in this study. *Malassezia* cells that were maintained on potato-dextrose agar (PDA) containing 1% yeast extract and a 1% peptone (YEP) slant in the presence of olive oil at 20 to 25 $^{\circ}\text{C}$ were spread on PDA-YE plates, layered with olive oil and cultured for 7 days at 30 $^{\circ}\text{C}$.

Crude antigens. *Malassezia* cells were washed four times in distilled water by centrifugation, suspended in 300 mM β -mercaptoethanol (2-ME) in 100 mM EDTA (pH 9.0) and then agitated for 60 min at 20 to 25 $^{\circ}\text{C}$. The resultant supernatants were dialyzed against distilled water and lyophilized. These fractions are referred to as the 2-ME extract.

Purification of 46 kDa and 67 kDa components of *M. globosa*. The 2-ME extract prepared from *M. globosa* NUM 6006 (200 mg) was dissolved in 10 ml of 10 mM Tris-HCl buffer column (pH 7.5) and applied to a DEAE Sephacel column (Pharmacia Biotech, Uppsala, Sweden). The column was washed with 10 mM Tris-HCl buffer (pH 7.5) until the A_{280} of the elute fractions was negligible. Materials that bound with the column were eluted with a linear gradient of 0 to 300 mM of NaCl in Tris-HCl buffer (pH 7.5). The A_{280} of all fractions was measured and the protein components in the fractions corresponding to each peak were distinguished by SDS-PAGE. A 46 kDa band was seen in two different fractions, and these fractions were designated as F1 and F2. The fraction containing a 67 kDa component was designated as F3. F2 was dialyzed against 10 mM acetate buffer (pH 4.5) and applied to a CM Sepharose Fast Flow column (Pharmacia Biotech) to remove additional components. The column was washed with 10 mM acetate buffer (pH 4.5) and materials that bound with the column were eluted with 10 mM acetate buffer contain-

ing 300 mM NaCl. The fractions containing a ca. 46 kDa component were pooled, and designated as F2b. In this study, the 46 kDa component that appeared as a major band in F1, the 46 kDa component as a major band in F2b and the 67 kDa component shown as a major band in F3 are referred to as Malg46a, Malg46b and Malg67, respectively.

SDS-PAGE and lectin blot. The procedure of SDS-PAGE was similar to that described by Ishiguro (5). Ten microliters of each sample solution was loaded on a 10% polyacrylamide gel. After electrophoresis, the gel was fixed with a mixture of acetic acid/methanol and Coomassie brilliant blue R (CBB) staining was performed to detect proteins in the samples.

For detection of mannans in the antigenic components, lectin blotting with concanavalin A (Con A) (from *Canavalia ensiformis*, specific for α -D-Man and α -D-Glc) was performed. The procedure of lectin blotting with Con A was similar to that described previously (9). After SDS-PAGE, the gel was electroblotted onto a polyvinylidene difluoride (PVDF) sheet (Millipore, Bedford, Mass., U.S.A.) and the sheet was soaked into 3% BSA in Tris-HCl containing 0.5 M NaCl (TBS) for 30 min at 20 to 25 $^{\circ}\text{C}$. The sheet was then incubated for 40 min at 20 to 25 $^{\circ}\text{C}$ with 4 $\mu\text{g}/\text{ml}$ of horseradish peroxidase (HRP)-conjugated Con A (Honen Co., Tokyo) in TBS containing 1% BSA. The sheet was finally incubated with a mixture of α -naphthol-TBS- H_2O_2 for 20 min at 20 to 25 $^{\circ}\text{C}$.

Detection of IgE antibodies to *M. globosa*. CAP-RAST (Pharmacia Diagnostics, Uppsala, Sweden) was also used to estimate the total IgE antibody level to *Malassezia* in each of the patients' sera. The UA value (kUA/liter) that was obtained from a standard curve using a reference serum for IgE (Pharmacia Diagnostics) indicated the level of IgE antibodies specific for *Malassezia* in the patient's serum (10).

For the Western blot, 10 μl of the sample solutions was loaded on 10% polyacrylamide gel and electroblotted onto PVDF sheets. The sheets were soaked in 20 mM Tris-HCl containing 0.5 M NaCl (TBS, pH 7.5) buffer with 3% skim milk for 30 min at 20 to 25 $^{\circ}\text{C}$ and then reacted with the patients' sera (1:20 in TBS containing 1% skim milk) overnight at 4 to 6 $^{\circ}\text{C}$. After several washes in 1% skim milk-TBS, each sheet was incubated with alkaline phosphatase (AP)-conjugated anti-human IgE (ϵ) goat serum diluted 1:2,000 in 1% skim milk in TBS for 3 hr at 21 to 25 $^{\circ}\text{C}$. The sheets were finally incubated in a mixture of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 mM Tris-HCl buffer (pH 9.5) that contained 100 mM NaCl and 5 mM MgCl_2 for 30 to 40 min at 20 to 25 $^{\circ}\text{C}$.

For the dot blot test, 1 ml of F2b (400 μg protein/ml)

was placed onto nitrocellulose (NC) sheets (Cellulose-nitrate, E BA 85; Schleicher & Schuell, Dassel, Germany) and dried for 30 min at 20 to 25 C. The sheets were blocked by soaking in 20 mM TBS containing 3% skim milk for 30 min. Each sheet was reacted with each of the patients' sera or control sera, which was diluted 1:20 in TBS containing 1% skim milk, overnight at 4 to 6 C. After washing in the buffer, the sheets were processed as described in the Western blot test for IgE antibodies.

Polyclonal antibody to *M. globosa*. F2b was loaded on a 10% polyacrylamide gel and the gel was stained with CBB. The 46 kDa band was removed and injected into rabbits with Freund's complete adjuvant (FCA) (Difco Lab, Detroit, Mich., U.S.A.). After additional injection of the 46 kDa component, rabbit serum was collected and used as an anti-Malg46b polyclonal antibody in the Western blot, which was performed to study the cross-reactivity of Malg46b among the *Malassezia* species.

Measurement of band or dot intensities generated for *M. globosa* antigens. To show the degree of reaction of the antigenic components of *Malassezia* with the IgE antibodies of the patients' sera, the intensities (arbitrary units) of bands in the Western blot or the dot intensities of F2b in dot blotting were measured with a Digital Image System IS 1000 instrument (Alpha Innotech Co., San Leandro, Calif., U.S.A.) (10).

Enzymatic treatments. F2b was dissolved in 200 mM citrate buffer (pH 5.0) (350 µg protein/ml) and was treated with 100 U/ml endoglycosidase H (Daiichi Pure Chemicals, Co., Ltd., Tokyo) or 10 U/ml of α -mannosidase (Daiichi Pure Chemicals) for 60 min at 37 C in 0.2 M citrate buffer (pH 5.0).

Measurement of proteins. The protein content of the antigenic fractions was measured by a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) and the protein content in each sample was adjusted to 400 µg protein /ml for SDS-PAGE and Western blotting.

Results

Fractionation and Purification of the Major Antigenic Components of *M. globosa*

The 2-ME extract of *M. globosa* contained several protein components, of which ca. 67 and 46 kDa components were seen as a major band in the extract (Fig. 1, lane 1). In the Western blot, IgE antibodies to the 67 and 46 kDa components were frequently seen in the sera of AD patients (Fig. 1, lane 2). This suggested that the 46 and 67 kDa components were possible major antigenic components of *M. globosa*. We thus isolated these components from the extract of *M. globosa*.

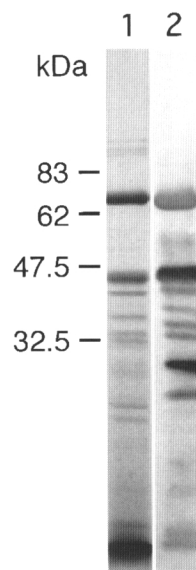


Fig. 1. CBB staining and Western blotting of the 2-ME extract of *M. globosa*. The 2-ME extract of *M. globosa* contained two major components, ca. 67 kDa and ca. 46 kDa in CBB staining (lane 1). IgE antibodies to these components were detected dominantly in the sera of patients with AD in the Western blot. Lane 2 is one of the examples that showed positive reactions. The mobilities of the molecular mass markers (in kilodaltons) are indicated on the left.

The 2-ME extract was applied to a DEAE Sephacel column and eluted with a linear gradient of 0 to 300 mM NaCl in 10 mM Tris-HCl. When the components of the eluted fractions were monitored on SDS-PAGE with CBB staining, a 46 kDa component was observed in two different fractions, which were eluted with approximately 70 mM NaCl (F1) and 240 mM NaCl (F2). The 67 kDa band was observed in the fractions which were eluted with 270 mM NaCl (F3). Since F2 contained several minor bands, F2 was further applied to a CM Sepharose column to remove the minor components from the fraction. The 46 kDa component was eluted with 300 mM NaCl in 10 mM citrate buffer and this fraction was designated as F2b. Each of the major components of the fractions was referred to as Malg46a for the 46 kDa band in F1 (Fig. 2, lane 2), Malg46b for the 46 kDa band in F2b (Fig. 2, lane 3) and Malg67 for the 67 kDa band in F3 (Fig. 2, lane 4).

Evidence That Malg46b Is a Major Antigen for IgE Antibodies in Patients with AD

To determine the antigenicity of the major components for IgE antibodies of the patients with AD, F1, F2b and F3 were electroblotted onto PVDF and incubated with the sera of the patients that were positive for IgE antibodies against the 2-ME extract of *M. globosa*. Malg46b and Malg67 reacted with approximately 70

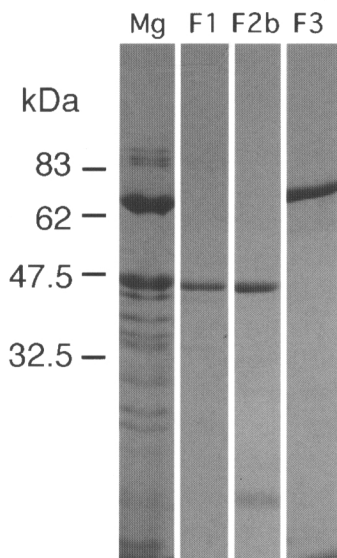


Fig. 2. CBB staining of antigen fractions. Both F1 and F2b contained a ca. 46 kDa band and F3 contained a ca. 67 kDa band as the major component, which are referred to as Malg46a for F1, Malg46b for F2b and Malg67 for F3. Mg indicates 2-ME extract of *M. globosa*. The mobilities of the molecular mass markers (in kilodaltons) are indicated on the left.

and 33%, respectively, of the sera tested but Malg46a did not react with any sera. These results indicated that Malg46b is the most antigenic of the major components.

F2b, therefore, was dotted onto NC sheets and the sheets were incubated with the sera from patients with AD, SD, CU, TV or healthy persons to study the specificity of this antigenic component to the skin diseases. F2b reacted only with the sera from patients with AD and none of the sera from the patients with SD, CU, TV or healthy persons showed positive reactions (Fig. 3A). In the dot blot test with F2b, F2b reacted with 69% of the sera of the patients with AD (Fig. 3B, column 1). When all sera containing IgE antibodies to *M. globosa* in the Western blot as shown in Fig. 1 were examined, F2b reacted with 83% (Fig. 3B, column 2).

The intensities of dots generated for F2b were measured by an IS1000 Image Analyzing System and compared with the total intensities of positive bands obtained from Western blotting, in which the 2-ME extract of *M. globosa* was used, or with the UA values (kUA/liter) obtained from CAP-RAST. The dot intensities of F2b correlated highly with the total intensities in the Western blot ($r=0.763$) (Fig. 4A), and the dot intensities also correlated with the UA values although the correlation was lower than that with the total intensities ($r=0.515$) (Fig. 4B).

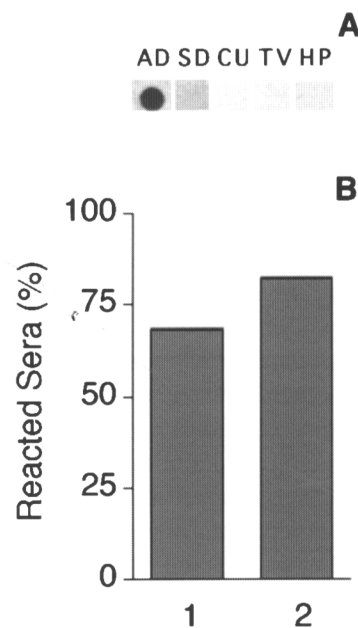


Fig. 3. Dot blot tests with F2b in sera from patients with various skin diseases. F2b was blotted onto NC sheets and the sheets were incubated with sera from patients with AD, SD, CU, TV or HP. IgE antibodies binding to F2b were seen only in the sera of patients with AD (A). Columns 1 and 2 show the positive percentage of sera reacted with F2b from AD patients and from the AD patients with IgE antibodies to *M. globosa*, respectively (B).

Characterization of Malg46b

As described above, the 46 kDa components (Malg46a and Malg46b) were fractionated into two distinct fractions. In a lectin blot with Con A, both Malg46a and Malg46b reacted with Con A (Fig. 5, lanes 2 and 3) but Malg67 did not (Fig. 5, lane 4). These results indicated that Malg46a and Malg46b were glycoproteins containing mannose chains in these molecules. To define the difference in antigenicity between Malg46a and Malg46b, a polyclonal antibody raised in rabbit against Malg46b was used in the Western blot. The polyclonal antibody reacted only with Malg46b (Fig. 6). To define the species specificity of Malg46b, 2-ME extracts which were prepared from various species of *Malassezia* were loaded on polyacrylamide gel (Fig. 7A) and electroblotted onto PVDF sheets for Western blotting with anti-Malg46b rabbit serum. The antibody reacted strongly with 3 strains of *M. globosa* (Fig. 7B-4). A slight reaction of the antibody was seen in the 2-ME extracts of two strains of *M. restricta* (Fig. 7B-3) but it did not react with the remaining three species of *Malassezia*.

To characterize epitopes of Malg46b for IgE antibodies, Malg46b was digested with endoglycosidase H. After enzymatic treatment, the molecular size of Malg46b was reduced to approximately 44 kDa (Fig. 8, lane 2) in the CBB stain, but Con A did not react with it

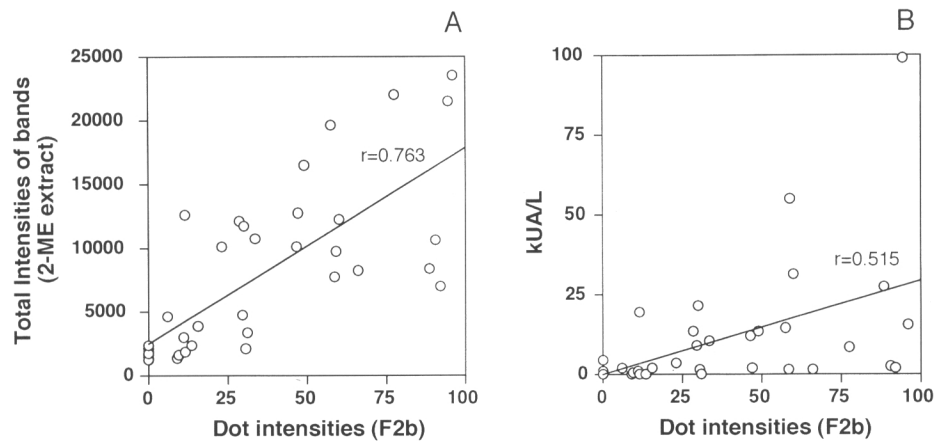


Fig. 4. Correlation of the dot intensities generated for F2b in the dot blot tests with the total intensities of bands generated for the 2-ME extract of *M. globosa* in Western blotting (4A) or with the UA scores of CAP-RAST (4B).

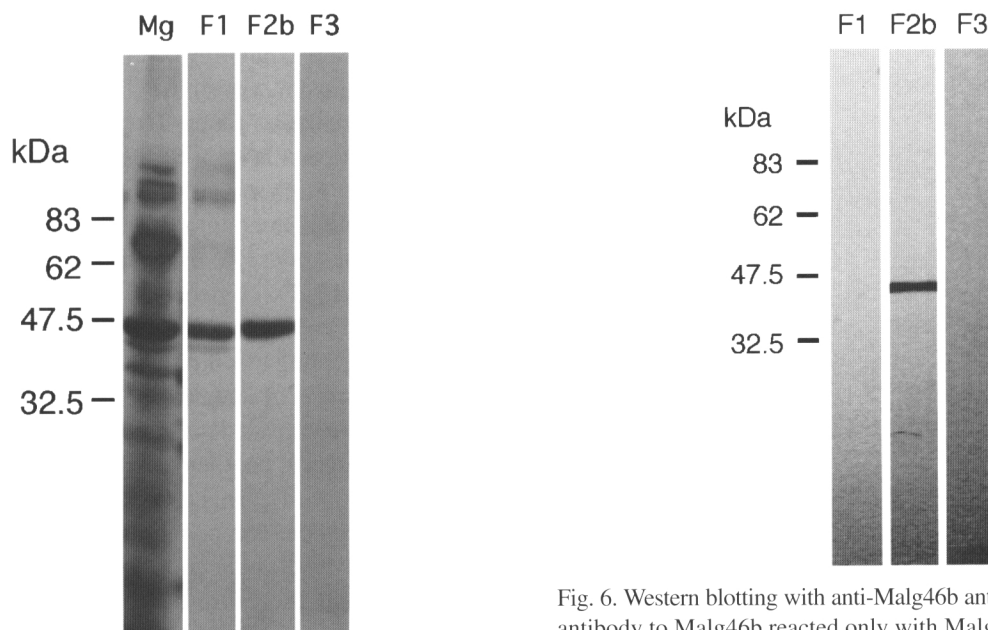


Fig. 5. Lectin blot with Con-A. 2-ME extract (Mg), F1, F2b and F3 were applied to SDS-PAGE, electroblotted onto a PVDF sheet and the sheet was incubated with HRP-Con A. Con A reacted with both Malg46a and Malg46b but not with Malg67. The mobilities of the molecular mass markers (in kilodaltons) are indicated on the left.

(Fig. 8, lane 4). When each of the sera of 12 AD patients with IgE antibodies to Malg46b was used for Western blotting with F2b after enzymatic treatment, all sera reacted with the 44 kDa band (Fig. 8, lane 6). Similar results were obtained when Malg46b was digested with α -mannosidase (data not shown).

Discussion

In this study, we defined a possible major antigen of *M. globosa* for IgE antibodies in patients with AD and

Fig. 6. Western blotting with anti-Malg46b antibody. A polyclonal antibody to Malg46b reacted only with Malg46b.

demonstrated its characteristics.

The 2-ME extract of *M. globosa* contained several components from which we isolated three major components by ion-exchange column chromatography, and these are referred to as Malg46a, Malg46b and Malg67 in this study. Both Malg46a and Malg46b showed the same profiles on mobility in SDS-PAGE and on reaction with Con A. However, the Western blot, in which the rabbit polyclonal antibody specific for Malg46b reacted only with Malg46b and not with Malg46a, showed that Malg46b differed serologically from Malg46a.

When IgE antibodies to Malg46a, Malg46b and Malg67 in patients with AD were scrutinized by Western blotting, IgE antibodies to Malg46b were detected in approximately 70% of the sera of the patients. This score was the highest among the major components examined. The dot blot test of F2b with the sera of all

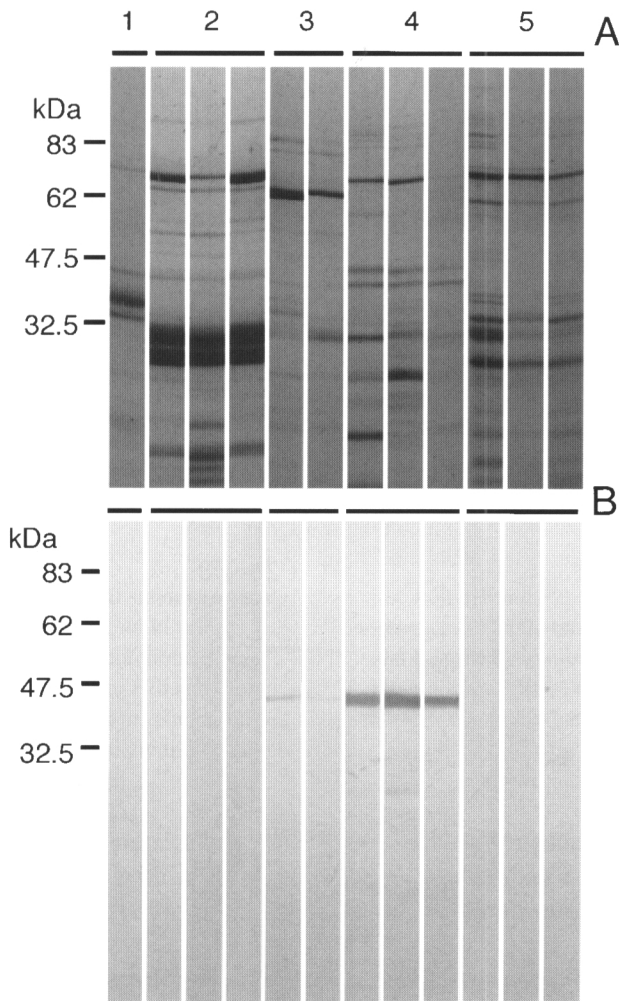


Fig. 7. Cross-reactivity of Malg46b among *Malassezia* species. Each bar corresponds to 1: *M. slooffiae*, 2: *M. sympodialis*, 3: *M. restricta*, 4: *M. globosa*, and 5: *M. furfur*, respectively. 2-ME extracts prepared from each species of *Malassezia* were applied to SDS-PAGE and stained with CBB. Species-dependent protein bands were seen (A). After SDS-PAGE, the gel was electroblotted onto a PVDF sheet and the sheet was incubated with an anti-Malg46b polyclonal rabbit serum (B).

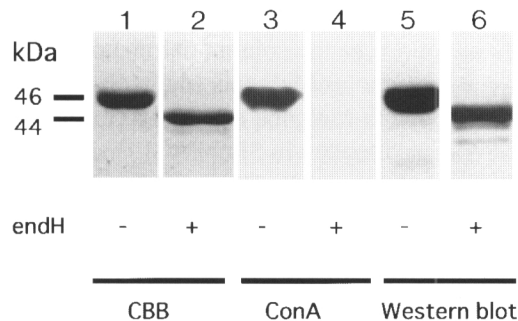


Fig. 8. Digestion of Malg46b with endoglycosidase H. After treatment with endoglucosidase H, the samples were applied to SDS-PAGE (lanes 1 and 2) and electroblotted onto PVDF sheets. The sheets were incubated with HRP-Con A (lanes 3 and 4) or sera containing IgE antibodies to Malg46b (lanes 5 and 6).

patients with AD and with the sera of the patients with AD containing IgE antibodies to the 2-ME extract of *M. globosa* in Western blotting showed that F2b reacted with 69 and 83% of the patients' sera, respectively. SDS-PAGE showed that F2b contained a major component of ca. 46 kDa (Fig. 2). Therefore, it is suggested that Malg46b is a major antigenic component for IgE antibodies in patients with AD. Furthermore, the dot intensities generated for F2b in the dot blot correlated highly with the total intensities generated for the 2-ME extract in the Western blot ($r=0.763$). This suggests that Malg46b was a major antigenic component in the extract. In our previous study, IgE antibodies of ca. 46 kDa were most frequently detected in Western blotting with the extract of *M. globosa* (manuscript submitted for publication), which is relevant to the results in the present study.

In the Western blot with polyclonal antibody to Malg46b obtained in this study, all strains of *M. globosa* showed the presence of a ca. 46 kDa component. This result indicated that Malg46b is species-specific but not strain-specific. Furthermore, this antibody reacted slightly with a ca. 46 kDa component of *M. restricta*, which suggested that *M. restricta* also has a low concentration of the ca. 46 kDa component. Several papers reported many possible major allergens of *M. furfur* (7, 8, 12, 15), however, Malg46b didn't fit any of the allergens reported. Huang et al studied the allergen components of *M. furfur* and reported a 46 kDa component as one of the important allergens, but the other allergens had stronger positive reactions (4). This is because the previous studies on the antigenic components of *M. furfur* were not based on the new classification for *Malassezia* species, and they might therefore reflect those of the other *Malassezia* species. A major protein allergen of *Candida albicans* is reported to be an enolase with a molecular weight ca. 46 kDa (6). However, a polyclonal antibody generated for *C. albicans* enolase did not react with the Malg46b in the Western blot (data not shown). More directly, when the sequence of 20 amino acids at the N-terminal of Malg46b was compared with the database, we obtained no homologous protein (unpublished data). These results suggested that Malg46b is a novel molecule expressed in *M. globosa* and *M. restricta*.

In the correlation study between F2b and CAP-RAST, the dot intensities for F2b correlated slightly with the UA values of CAP-RAST ($r=0.515$); the correlation was much lower than that between F2b and the 2-ME extract of *M. globosa* ($r=0.763$). This low correlation of F2b with CAP-RAST can be explained as follows. UA values of CAP-RAST correlate highly with the band intensities of *M. sympodialis* ($r=0.917$) rather than with those of *M. globosa* ($r=0.590$) (manuscript submitted for publi-

cation). Thus, the UA values of CAP-RAST seem to reflect the IgE level to *M. sympodialis*, not to *M. globosa*. As described above, Malg46b, which is a major component in F2b, is expressed highly in *M. globosa* but not expressed in *M. sympodialis*.

IgE antibodies to F2b were detected only in the sera from patients with AD, and not in any sera from patients with SD, CU or TV, despite the fact that the *Malassezia* species were isolated from the skin of patients with SD and TV (unpublished data). These results suggested that IgE antibodies for Malg46b were generated specifically in patients with AD. To confirm the specificity of Malg46b in these skin diseases, a greater number of patients' sera should be collected.

The lectin blot, in which F2b was incubated with Con A, showed that Malg46b is a glycoprotein of ca. 46 kDa. Enzymatic digestion of F2b with endoglycosidase H or α -mannosidase generated a single band of ca. 44kDa, indicating that Malg46b is a glycoprotein. In Western blotting, all the sera of patients with AD (12 sera) reacted with the 44 kDa band, suggesting that the protein portion of Malg46b contains epitopes for IgE antibodies in the patients' sera.

In conclusion, a 46 kDa glycoprotein, Malg46b, showed high species specificity to *M. globosa* and constituted a major antigenic component of this fungus in patients with AD. In order to define the role of Malg46b as an allergen in AD, the ability to release histamine, produce interleukine and cause skin reactions with this molecule are now under investigation.

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References

- 1) Broberg, A., and Faergemann, J. 1995. Topical antimycotic treatment of atopic dermatitis in the head/neck area. A double-blind randomised study. *Acta Derm. Venereol.* **75**: 46-49.
- 2) Gueho, E., Midgley, G., and Guillot, J. 1996. The genus *Malassezia* with description of four new species. *Antonie van Leeuwenhoek* **69**: 337-355.
- 3) Guillot, J., Gueho, E., Lesourd, M., Midgley, G., Chevrier, G., and Dupont, B. 1996. Identification of *Malassezia* species. A practical approach. *J. Mycol. Med.* **6**: 103-110.
- 4) Huang, X., Johansson, S.G.O., Zargari, A., and Nordvall, S.L. 1995. Allergen cross-reactivity between *Pityrosporum orbiculare* and *Candida albicans*. *Allergy* **50**: 648-656.
- 5) Ishiguro, A., Homma, M., Torii, S., and Tanaka, K. 1992. Identification of *Candida albicans* antigens reactive with immunoglobulin E antibody of human sera. *Infect. Immun.* **60**: 1550-1557.
- 6) Ito, K., Ishiguro, A., Kanbe, T., Tanaka, K., and Torii, S. 1995. Characterization of IgE-binding epitopes on *Candida albicans* enolase. *Clin. Exp. Allergy* **25**: 529-535.
- 7) Jensen-Jarolim, E., Poulsen, L.K., With, H., Kieffer, M., Ottevanger, V., and Skov, P.S. 1992. Atopic dermatitis of the face, scalp, and neck: type 1 reaction to the yeast *Pityrosporum ovale*? *J. Allergy Clin. Immunol.* **89**: 44-51.
- 8) Johansson, S., and Karlstrom, K. 1991. IgE binding components in *Pityrosporum orbiculare* identified by an immunoblotting technique. *Acta Derm. Venereol.* **71**: 11-16.
- 9) Kanbe, T., Han, Y., Redgrave, B., Riesselman, M.H., and Cutler, J.E. 1993. Evidence that mannans of *Candida albicans* are responsible for adherence of yeast forms to spleen and lymph node tissue. *Infect. Immun.* **61**: 2578-2584.
- 10) Kanbe, T., Morishita, M., Ito, K., Tomita, K., Utsunomiya, K., and Ishiguro, A. 1996. Evidence for the presence of immunoglobulin E antibodies specific to the cell wall phosphomannoproteins of *Candida albicans* in patients with allergies. *Clin. Diagn. Lab. Immun.* **3**: 645-650.
- 11) Leeming, J.P., Sansom, J.E., and Burton, J.L. 1997. Susceptibility of *Malassezia furfur* subgroups to terbinafine. *Br. J. Dermatol.* **137**: 764-767.
- 12) Lintu, P., Savolainen, J., and Kalimo, K. 1997. IgE antibodies to protein and mannan antigens of *Pityrosporum ovale* in atopic dermatitis patients. *Clin. Exp. Allergy* **27**: 87-95.
- 13) Mayser, P., Haze, P., Papavassilis, C., Pickel, M., Gruender, K., and Gueho, E. 1997. Differentiation of *Malassezia* species: selectivity of Cremophor EL, castor oil and ricinoleic acid for *M. furfur*. *Br. J. Dermatol.* **137**: 208-213.
- 14) Nordvall, S.L., and Johansson, S. 1990. IgE antibodies to *Pityrosporum orbiculare* in children with atopic disease. *Acta Paediatr. Scand.* **79**: 343-348.
- 15) Savolainen, J., and Broberg, A. 1992. Crossreacting IgE antibodies to *Pityrosporum ovale* and *Candida albicans* in atopic children. *Clin. Exp. Allergy* **22**: 469-474.
- 16) Simmons, R.B., and Gueho, E. 1990. A new species of *Malassezia*. *Mycology* **94**: 1146-1149.
- 17) Tagami, H. 1996. Japanese Dermatological Association Criteria for the diagnosis of atopic dermatitis. *NIHON-HIFU-KA-GAKKAI-ZASSHI* **106**: 64-65.
- 18) Wessels, M.W., Doekes, G., Dijk, A.G., et al. 1991. IgE antibodies to *Pityrosporum ovale* in atopic dermatitis. *Br. J. Dermatol.* **125**: 227-232.