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Isolation and Biochemical Characterization of the iC3b Receptor of *Candida albicans*

S. ALAEI, C. LARCHER, C. EBENBICHLER, W. M. PRODINGER, J. JANATOVA,† AND M. P. DIERICH*

Institut für Hygiene, University of Innsbruck, Innsbruck, Austria

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In an effort to identify the protein structure on *Candida albicans*, pseudohyphal forms which had been shown earlier to bind human iC3b, a protein of about 42 kDa (p42), were obtained from lysates of pseudohyphal forms by absorption with C3(H_2O)-Sepharose. An antiserum raised in rabbits against this protein effectively inhibited adherence of sheep erythrocytes carrying iC3b (EAC3bi) to pseudohyphal forms. p42 cross-reacted with OKM-1, a monoclonal antibody directed against the human complement receptor type 3 (CR3, CD11b). This protein, p42, was designated p42-CR3. The antiserum against p42-CR3 was used for further purification of lysates by affinity chromatography. Three proteins of 66, 55, and 42 kDa were isolated. All were recognized by OKM-1 in immunoblots (p66-, p55-, and p42-CR3). The different proteins were separated and treated with neuraminidase and endoglycosidase F. Almost complete deglycosylation of the p66-CR3 protein was obtained after treatment with neuraminidase, indicating a high degree of glycosylation. Neuraminidase also had an effect on p55-CR3, but not on p42-CR3. Endoglycosidase F did not alter any of the three proteins. In ligand blots, p42-CR3 bound C3(H_2O), C3b, and iC3b but not C3d; p55-CR3 clearly reacted with C3(H_2O) and weakly reacted with C3b and iC3b. p66-CR3 never showed reactivity. It is suggested that p55 and p66 represent glycosylated forms of p42-CR3. Although *C. albicans* CR3 and human CR3 cross-react and bind identical ligands, the two receptors differ in structure.

Candida albicans pseudohyphal forms, in contrast to yeast forms, bind sheep erythrocytes carrying iC3b or C3d (EAC3bi, EAC3d). This observation, initially described by Heidenreich and Dierich (10), was confirmed later by several other investigators (3, 4, 6).

Eigentler et al. (5) have shown that the expression of the iC3b receptor on *C. albicans* is dependent on the growth temperature. *C. albicans* grown at 30°C bound EAC3bi strongly, whereas those cells grown at 38.5°C were completely devoid of that feature.

The C3d-binding proteins of *C. albicans* were purified by ion-exchange and affinity chromatography. Two proteins with molecular masses of 62 and 70 kDa were identified by silver stain, by Western blots (immunoblots) with CA-A, a monoclonal antibody against *C. albicans* that inhibited interaction of *C. albicans* with EAC3d, and by size exclusion high-pressure liquid chromatography (2, 3, 11). Ollert et al. (12) reported data on a human serum which inhibited the iC3b-mediated adherence almost completely; this serum reacted with 71-, 68-, 55-, and 50-kDa molecules in immunoblots of extracts *C. albicans* pseudohyphae.

Here we report on the isolation of iC3b binding molecules from *C. albicans*.

MATERIALS AND METHODS

Reagents. OKM-1, a mouse monoclonal antibody of the immunoglobulin G2b (IgG2b) subtype directed against the alpha chain of human complement receptor type 3 (CR3, CD11b), was purchased from Ortho Diagnostic Systems, Raritan, N.J. C3, C3(H_2O), and the C3 fragments C3b, iC3b, and C3d were prepared in our laboratory according to standard methodology (1, 7–9).

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Samples were run on sodium dodecyl sulfate (SDS)-9.5% polyacrylamide gels under reducing conditions. After electrophoresis was completed, the gels were stained with either Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, Calif.) or silver. From parallel gels, proteins were electrophoretically transferred to nitrocellulose paper (0.45-μm pore size; Schleicher & Schuell, Dassel, Germany). The blots were saturated for 20 min in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 1% skim milk, 0.5% gelatin, and 0.1% Triton X-100 or for 2 h in PBS containing 0.5% gelatin and 0.1% Triton X-100 (PGt) at 20°C, washed three times for 10 min with PGt, and incubated with the first antibody diluted in PGt for 1 to 2 h at 20°C. After being washed two times with PGt, the blots were incubated with the second peroxidase-conjugated antibody (Dakopatts, Glostrup, Denmark) for 1 h at 20°C. After being washed once with PGt and once with PBS containing 0.05% Tween 20, blots were developed with diaminobenzidine by standard procedures.

Organism and cultivation. *C. albicans* CBS 5982 (serotype A) was used throughout these studies and has been described previously (5). The organism was grown on Sabouraud dextrose agar (Oxoid, Basingstoke, Hampshire, England) at 37°C for 24 h. Pseudohyphal forms were raised by a two-stage cultivation procedure. RPMI 1640 medium (150 ml) (Flow Laboratories, Meckenheim, Germany) in 1-liter Erlenmeyer flasks was inoculated with *C. albicans* from an agar plate and incubated for 16 h at 30°C while rotating at 150 rpm. This seed culture was transferred to fresh RPMI 1640 medium (inoculum size, 8% of total volume), and cultivation was continued under the same conditions for 15 to 24 h. Alternatively, the second stage of cultivation was performed at 30°C in RPMI 1640 medium in a 10-liter fermentor after

* Corresponding author.
† Present address: Artificial Heart Research Laboratory, Institute for Biomedical Engineering, University of Utah, Salt Lake City, UT 84112.

inoculation with the seed culture (8% of total volume). To avoid cell damage by shearing, this culture was aerated (9 liters/min) without any mechanical stirring.

Pseudohyphal growth was observed by determination of dry cell weight, DNA content, and microscopic control. At least 90% of the cell mass obtained by the cultivation described above was of pseudohyphal form.

Complement-coated sheep erythrocyte (EAC) intermediates. Sheep erythrocytes were coated with complement components as described previously (5). Briefly, sheep erythrocytes were sequentially exposed to C1, oxidized C2, and hemolytically active C3. Cleavage of erythrocyte-bound C3b to iC3b by factors H and I was achieved by incubation with heat-inactivated human serum at 37°C for 30 min.

Adherence assay. *C. albicans* CBS 5982 cultivated in RPMI 1640 medium as described above was washed with PBS and adjusted to a density of 2×10^7 pseudohyphae per ml of PBS. Samples (50 μl) of this suspension were incubated with 50 μl of EAC suspension adjusted to 1.4×10^9/ml of PBS. After incubation for 30 min at 37°C, the attachment index was determined by counting the number of erythrocytes adhering per 30 pseudohyphae and then dividing the resulting number by 30.

To measure adherence inhibition by isolated iC3b receptor material (*C. albicans* CR3), EACs were preincubated with potential inhibitors for 30 min at 37°C, washed once with PBS, and then reacted with pseudohyphal forms as described above.

Preparation of extracts from pseudohyphal forms of *C. albicans*. Pseudohyphal forms were centrifuged ($600 \times g$ for 10 min), washed with PBS, and resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride (E. Merck AG, Darmstadt, Germany), 0.01 mM leupeptin (Boehringer GmbH, Mannheim, Germany), and 0.1 mM E-64 (Boehringer) at a concentration of 0.2 to 0.6 mg (wet weight) per ml. A 5-ml portion of this suspension was transferred to a 50-ml glass tube (Corex), 10 g of glass beads with a diameter of 0.45 to 0.5 mm (Sigma Chemical Co., St. Louis, Mo.) was added, and the cells were disrupted by high-speed mixing on a vortex mixer (REAX 2000; Heidolph, Kehlheim, Germany) with intermittent ice bath cooling every 30 s (total mixing time, 10 min). Subsequently, cell debris was removed by centrifugation at $10,000 \times g$ and $25,000 \times g$, each for 20 min, and $100,000 \times g$ for 1 h. The clear final supernatant was used for the following purification steps.

Affinity chromatography with C3(H_2O) coupled to CNBr-activated Sepharose. The coupling of 1.5 to 2 mg of C3(H_2O) to 1 ml of CNBr-activated Sepharose [C3(H_2O)-Sepharose; Pharmacia, Uppsala, Sweden] was done according to the instructions of the manufacturer. Incubation was performed for 1.5 h at room temperature. This C3(H_2O)-Sepharose was washed three times in 5 to 10 ml of PBS and thereafter suspended in 5 ml of PBS and stored at 4°C. BSA-Sepharose was prepared in the same manner by coupling 2 mg of BSA (Sigma A-9647) to 1 mg of swollen CNBr-Sepharose.

Adsorption of receptor material to C3(H_2O)-Sepharose. *C. albicans* extract (1 ml; total protein concentration, 1 mg of protein per ml) was mixed with 500 μl of BSA-Sepharose for 2 h at 4°C. After centrifugation ($600 \times g$ for 10 min), this preabsorbed supernatant was divided into two equal portions. One was incubated with 100 μl of C3(H_2O)-Sepharose for 2 h at 4°C. After centrifugation ($1,500 \times g$ for 10 min), the pellet was washed three times with PBS and the proteins were eluted with 1 ml of 0.4 M NaCl solution. This material was used for the adherence assay and analysis by SDS-PAGE. A total of 50 μl was mixed with 50 μl of Tris-HCl buffer (1 M, pH 7.8) containing 2% mercaptoethanol and boiled for 5 min. The resulting supernatant was analyzed by SDS-PAGE and immunoblot. Blots were incubated with OKM-1 diluted 1:100 (concentration of stock solution, 200 μg/ml). The second portion was again incubated with BSA-Sepharose in the same manner as described above for C3(H_2O)-Sepharose. Incubation and further treatment were done as described above.

Preparation of antisera against *C. albicans* CR3 material in rabbits. On SDS-PAGE and subsequent Western blots, 42- and 70-kDa material obtained from C3(H_2O)-Sepharose absorption was identified as reactive with OKM-1. Gel bands containing this material were cut out, and each was layered on top of polyacrylamide gels run under nondenaturing conditions. These gels were stained with Coomassie blue devoid of acetic acid, and appropriate bands were cut out and mixed for the first immunization of chinchilla bastard rabbits with complete Freund's adjuvant and for the three subsequent subcutaneous boost injections (intervals of 2 weeks) with incomplete Freund's adjuvant. From the serum of the rabbit immunized with 42-kDa protein (serum 42), the IgG fraction was purified with a protein G-Sepharose kit (MAb TrapG; Pharmacia) according to the instructions of the manufacturer.

Affinity chromatography with purified rabbit IgG against *C. albicans* CR3 (serum 42). Binding of rabbit IgG against *C. albicans* CR3 to CNBr-Sepharose (Pharmacia) was performed in the same manner as described above for the coupling of C3(H_2O) to CNBr-Sepharose. In preliminary experiments, it was determined that 1 ml of coupled Sepharose had the capacity to bind quantitatively CR3 material from 9 mg of *C. albicans* pseudohyphal extract. The column (bed volume, 2 ml) was loaded with 3 ml of extract, washed with PBS, and eluted with 0.5 M glycine buffer at pH 2.7. Fifteen fractions of 1 ml were collected, and the pH was adjusted to 7.0 with 100 μl of 1 M Tris buffer (pH 9.0). Fractions were tested for protein concentration, for inhibitory activity in the adherence assay, and by immunoblot for reactivity with OKM-1 and rabbit anti-*C. albicans* serum 42. Additionally, the material eluted from the column was subjected to SDS-PAGE, the gel was stained with a Coomassie blue solution devoid of acetic acid, and the different proteins were isolated by electroelution of cut bands at 100 V for 3 h.

Deglycosylation. A 1-μl sample of a 20% SDS solution was added to 200 μl (approximately 200 μg of total protein) of the positive fractions resulting from anti-*C. albicans* CR3 affinity chromatography, boiled for 5 min, and then microdialyzed (Pierce) against 50 mM sodium acetate containing 10 mM EDTA and 0.5% Triton X-100 (pH 5.5). β-Mercaptoethanol (1 μl) was added, and the aliquots were then treated with 0.5 U of endoglycosidase F (endo F) (EC 3.2.1.96; Boehringer), 0.5 U of neuraminidase (EC 3.2.1.18) type V (Sigma), 0.5 U of endo F plus 0.5 U of neuraminidase, or with the corresponding buffer as a control for 8 or 20 h at 37°C. The effect of the incubation was tested by immunoblotting with OKM-1 and serum 42. The isolated proteins obtained by SDS-PAGE and electroelution were treated with the above-mentioned enzymes in the same manner.

Ligand blot. The fractions obtained by affinity chromatography were run on SDS-PAGE and transferred to nitrocellulose. Blot strips were incubated with 100-μg/ml solutions of C3(H_2O), C3b, iC3b, C3d, factor H, and a mixture of SDS-PAGE high- and low-molecular-weight standards (Bio-Rad) for 30 min at 37°C. After being washed, blots were incubated with anti-C3c and anti-C3d antibody (Dakopatts)

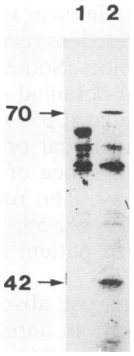

FIG. 1. Reaction of *C. albicans* in an immunoblot with OKM-1. Whole-cell extract after absorption with BSA-Sepharose (lane 1) and C3b-Sepharose (lane 2) is shown. Numbers on left show molecular mass in kilodaltons.

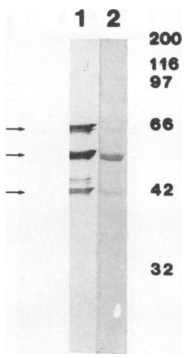

FIG. 2. Fraction 5 after affinity chromatography (10 μg of total protein) in an immunoblot against serum 42, dilution 1:100 (lane 1), and against OKM-1 (lane 2). Numbers on right show molecular mass in kilodaltons.

and then with peroxidase-conjugated second antibodies (Dakopatts).

RESULTS

Affinity chromatography with C3(H_2O)-Sepharose. The material obtained by desorption from C3(H_2O)-Sepharose with a 0.4 M NaCl solution had 50% inhibitory capacity in the iC3b adherence inhibition assay, while the material eluted from BSA-Sepharose showed no inhibition, suggesting the absence of complement receptor material in the eluate from the latter column. In Western blots, OKM-1 reacted with several bands. In addition to proteins present in both eluates, two proteins of 42 and 70 kDa were detected in the C3(H_2O)-Sepharose eluate (Fig. 1). Since the eluate of this column inhibited iC3b adherence, these two proteins were used for immunization of rabbits. The rabbit serum resulting from immunization with the 42-kDa protein (serum 42) had 46% (standard deviation, 14.1%) inhibitory activity and serum 70 from immunization with the 70-kDa protein had 50% inhibitory activity in the adherence inhibition assay when applied in a dilution of 1:100.

Since the 42-kDa protein was particularly distinct in Western blots, the IgG fraction of the antiserum produced against it was used for further purification.

Affinity chromatography with serum 42-Sepharose. Lysates of pseudohyphae were run over serum 42-Sepharose. Of the 15 fractions collected, only fraction 5 had a clear inhibitory effect (40 to 50%) in the iC3b adherence assay and reacted in Western blots with serum 42 diluted 1:100; three proteins (66, 55, and 42 kDa) were stained. OKM-1 regularly detected two proteins (55 and 42 kDa) and in some experiments detected all three bands (Fig. 2).

Effect of endo F and neuraminidase on isolated proteins. After the three proteins were separated by PAGE and electroelution, they were incubated with neuraminidase. The reaction pattern was seen in immunoblots with rabbit serum 42 (Fig. 3). The 66-kDa protein was partially deglycosylated after 8 h of incubation (Fig. 3, lane 2) to a protein of approximately 50 kDa and after 20 h (lane 3) to a 42-kDa protein and two additional proteins between 55 and 66 kDa. The 55-kDa protein almost completely disappeared after 20 h of incubation with neuraminidase (Fig. 3, lane 5), but no distinct deglycosylation product was detectable. Serum 42 stained a broad band between 55 and 66 kDa, possibly an aggregation product. The 42-kDa protein remained virtually unaffected by neuraminidase, with a slightly broader band after treatment (Fig. 3, lane 7). In this case also a possible aggregation product was visible between 55 and 66 kDa.

With OKM-1, only weak bands of 42 and 55 kDa were detectable, and no band was visible at 66 kDa after treatment with neuraminidase (data not shown). Treatment with endo F had no effect on the 42-, 55-, and 66-kDa proteins.

Reaction of *C. albicans* CR3 material with C3 fragments. The two fractions from the anti-42-kDa protein affinity chromatography (containing the 42-, 55-, and 66-kDa proteins) were incubated with C3(H_2O), C3b, iC3b, and C3d, with factor H, and with a mixture of proteins typically used as marker proteins, all at a concentration of 100 μg/ml. With C3(H_2O), two proteins (55 and 42 kDa) were visible in the ligand blot. With C3b and iC3b, one protein corresponding to 42 kDa was visible. The latter protein bound to a small extent to C3d, too. The controls with factor H and the marker proteins were negative (Fig. 4).

DISCUSSION

A single-chain protein of 42 kDa, p42, was isolated by C3(H_2O)-Sepharose absorption from pseudohyphal forms of *C. albicans* and used to raise an antibody which proved to inhibit adherence of iC3b-coated erythrocytes to pseudohy-

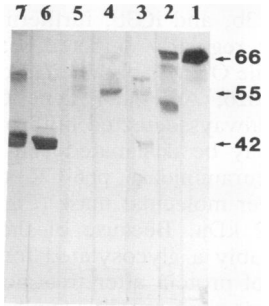

FIG. 3. Effect of neuraminidase on the isolated proteins of 42, 55, and 66 kDa (numbers on right show locations) as demonstrated in immunoblots with serum 42. The 66-kDa protein is shown untreated (lane 1) and after treatment with neuraminidase for 8 h (lane 2) and for 20 h (lane 3). The 55-kDa protein is shown untreated (lane 4) and after treatment with neuraminidase for 20 h (lane 5). The 42-kDa protein is shown untreated (lane 6) and after treatment with neuraminidase for 20 h (lane 7).

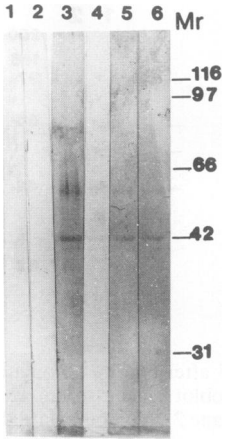

FIG. 4. Ligand blots with positive fractions from affinity chromatography. Reactions with C3(H_2O), C3b, iC3b, and C3d and controls with factor H or buffer containing marker proteins instead of any possible ligand: lane 1, factor H, stained with anti-H; lane 2, buffer control, stained with anti-C3c; lane 3, C3(H_2O), stained with anti-C3d; lane 4, C3d, stained with anti-C3d; lane 5, C3b, stained with anti-C3c; lane 6, iC3b, stained with anti-C3c.

phal forms. p42 was recognized by OKM-1, a monoclonal antibody against the alpha chain of the human iC3b receptor, CR3. It bound soluble C3(H_2O), C3b, and iC3b well, but bound only weakly to C3d. For these reasons, it is called p42-CR3. This designation of p42 is further supported by preliminary experiments showing a cross-reaction of the anti-p42-CR3 with human CR3 on U937 cells but not with CR2 on Raji cells. These data resulted from standard immunofluorescence assays comparing serum 42 with the preimmune serum (dilution, 1:500).

Upon treatment with neuraminidase, the molecular mass of p42 could not be reduced, suggesting that p42-CR3 does not contain sialic acid residues. Since endo F did not remove any carbohydrate either alone or in conjunction with neuraminidase, and Calderone et al. (3) used binding by concanavalin A as a criterion for purification of complement receptors from *C. albicans*, the lack of carbohydrate could be the reason for their failure to detect p42-CR3.

The antiserum against p42-CR3 typically precipitated two additional proteins of 55 and 66 kDa. p55-CR3 also reacted with C3(H_2O), C3b, and iC3b; furthermore, commercially available OKM-1 regularly detected p55 and occasionally detected p66, while OKM-1 obtained in our laboratory from a clone (CRL 8026; American Type Culture Collection, Rockville, Md.) always detected both proteins. Therefore, these proteins may be designated p55- and p66-CR3. By treatment with neuraminidase, p66-CR3 could be reduced to molecules of lower molecular mass (Fig. 3), including species of about 42 kDa. Because of these characteristics, p66-CR3 is probably a glycosylated form of p42-CR3, although the loss of protein after treatment with neuraminidase is not understood. A possible explanation might be that after loss of sialic acid, proteins become more sticky and are not completely transferred from the deglycosylation assay tube. In addition, it is conceivable that even after 20 h, many intermediate deglycosylation products which are distributed over a broad molecular mass range exist. These would not be detected even by Western blot because of the low concentration of each molecular mass species.

p55-CR3 upon neuraminidase treatment tended to form larger complexes that were less recognizable by the anti-p42-CR3 serum in Western blots. Some of these complexes seem to be similar to material obtained after 20 h of treatment of p66-CR3 with neuraminidase. p55 might be a protein related to p42, sharing some identical or cross-reactive epitopes. This could explain the absence of the 42-kDa species after the deglycosylation assay. The relationship of these three forms to molecules of 71, 68, 55, and 50 kDa identified by Ollert et al. (12) using a patient's serum is presently not clear.

During C3(H_2O)-Sepharose absorption, a 70-kDa protein was also isolated (Fig. 1); an antiserum against it inhibited iC3b adherence. A protein of this size was also obtained by ion-exchange chromatography and inhibited iC3b adherence (data not shown). Since the anti-p42-CR3 serum never precipitated this molecular species, either it is a type different from p42, p55, and p66 or it has the same protein backbone but a higher degree of glycosylation covering the sites recognized by anti-p42-CR3.

Although p42-, p55-, and p66-CR3 do react with OKM-1 and show binding characteristics like those of human CR3, they obviously have a very different structure. While human CR3 has an alpha chain and a beta chain, in *C. albicans* CR3, a heterodimer was not observed. With p42-CR3 now available, the molecule's structure can now be clarified.

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