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The iC3b receptor of *Candida albicans* and its Roles in Pathogenesis

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Abstract

On the basis of biochemical and immunologic studies, a receptor for iC3b with some activities reminiscent of the integrins CD11b and CD11c was defined on the cell wall of clinical and laboratory isolates of *Candida albicans*. The *INT1* gene encodes a protein of 1659 amino acids; the Int1 protein participates in adhesion to epithelial cells in vitro and in vivo. Int1 is essential for hyphal morphogenesis and virulence in a murine model. Recent evidence points to the amino terminus of Int1 as the source of a peptide, Pep₂₆₃, with superantigen-like activities.

Biochemistry of Complement-Mediated Opsonization

Studies from the laboratory of Brian Tack were among the first to characterize the molecular mechanism by which the third component of complement, C3, binds covalently to free hydroxyl or amino groups. The glutamyl residue of the thiolester peptide ¹⁰⁰⁹Gly-Cys-Gly-Glu-Gln₁₀₁₃ donates its carbonyl group in a transacylation reaction to form either an oxy-ester bond with carbohydrate acceptors or an amide bond with amino acceptors. In the absence of such acceptors, the thiolester site binds water at the glutamyl carbonyl, forming carboxylic acid and rendering the protein incapable of further covalent interaction [1].

The internal thiolester bond of C3 links Cys₁₀₁₀ and Gln₁₀₁₃ residues within the labile binding site of native C3, which is situated within the C3d subdomain of the α -chain of C3. This site is exposed when convertases of the classical, MBL, or alternative pathways cleave the C3a moiety from the amino terminus of the C3 α chain, and within the next 60 microseconds, the reactive glutamyl carbonyl in C3b is able to bind to carbohydrate or amino acceptors (Figure 1).

These reactions were shown to be applicable to the opsonization of *Streptococcus pneumoniae* [2] when Hostetter and colleagues demonstrated that reconstitution of the alternative complement pathway by the addition of purified C3 to human serum in which C3 and C4 were inactivated led to phagocytosis of 50-80% of pneumococcal serotypes 4, 6A, 14, or 18C, while the addition of preformed C3b (carboxylic acid at the reactive glutamyl carbonyl) or methylamine-treated C3 (amide bond occupying the reactive glutamyl carbonyl) resulted in

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phagocytosis of less than 7%. Once covalently bound to the pneumococcal surface, C3b was cleaved by factors H and I to generate the fragments iC3b and C3d, depending upon the pneumococcal serotype [3]. These results were generalized to group A streptococcus, *E. coli*, *Staphylococcus aureus*, and a number of other organisms [4-7].

Identification of an iC3b Receptor on *Candida albicans*

However, when *Candida albicans* was chosen as the organism to be opsonized, deposition of iC3b was non-covalent, like the reversible binding of iC3b to neutrophil complement receptor type 3 (CR3) [8,9]. In *C. albicans*, non-covalent binding of iC3b inhibited phagocytosis, presumably because non-covalent binding of iC3b obscured the ligand binding site recognized by CR3 [Figure 1]. Complement receptor type 3 is also known as the integrin $\alpha M/\beta 2$ or CD11b/CD18. Binding of monoclonal antibodies recognizing the α -chain (OKM1, Mo1, M1/70), but not the β chain of $\alpha M/\beta 2$ suggested the expression of putative CR3-like receptors on the surface of clinical isolates of *C. albicans* [10]. Monoclonal antibodies against CR1 and CR2 did not bind to *Candida albicans* [9] [Figure 2].

The binding of [^3H] iC3b was specific, saturable, and readily reversible in the presence of a 150-fold molar excess of unlabeled C3 [Figure 3] [9]. For two candidal isolates, the association constant (K_a) was similar at $2.45 \times 10^6 \text{ L/M}$, a determination very nearly identical to the K_a for the binding of iC3b to neutrophil CR3 [11]. In addition, expression of the putative receptor was significantly increased by hyphal transformation and by growth in the presence of 20-50 mM D-glucose [9]. Blockade of the putative candidal receptor by the IgM monoclonal antibody Mo1 increased phagocytosis significantly [9]. Subsequently, a number of monoclonal antibodies recognizing receptors for iC3b [9,12-14] were shown to bind to *C. albicans*, suggesting that protein(s) on the *C. albicans* surface might share some structural homology with the α -chains of the leukocyte integrins CD11b/CD18 or Cd11c/CD18 (CR4 or $\alpha X/\beta 2$).

The α -chains of αM and αX have masses of 165 and 150 kDa, respectively, and share approximately 70% sequence homology, as well as an inserted or I domain of approximately 200 amino acids that is involved in ligand binding [15,16]. In addition, located just C-terminal to the I domain in $\alpha M/\alpha X$ are three divalent cation binding sites distinguished by the consensus sequence DXNS [17]. Distal to the transmembrane domain at the C terminus is a cytoplasmic tail with a single tyrosine residue [16]. Immunoprecipitation with ^{125}I -labeled OKM1 or Western blotting with the monoclonal antibody Mo1 detected bands at Mr 130-165 kDa in extracts of cell wall, cell membrane, and cytosol from *C. albicans* blastospores [8,18].

Role of the iC3b Receptor in Candidal Adhesion

The antigenic relationship between putative iC3b receptors on *Candida albicans* yeast cells and the leukocyte integrin α -chains CD11b or CD11c extended to functional similarities as well. CD11b/CD18 and its analog in *C. albicans* had an identical affinity for iC3b, and increased binding of monoclonal antibodies against CD11b occurred in both neutrophils and in yeast cells exposed to temperatures of 37°C.

Other conditions that increased expression of iC3b receptors in *C. albicans* (e.g. growth on 20 mM D-glucose as compared to 20 mM L-glutamate) augmented adhesion of yeast cells to human umbilical vein endothelium, as measured by flow cytometry with monoclonal antibodies recognizing CD11b increased significantly [19]. Blockade of *C. albicans* iC3b receptors with IgM monoclonal antibodies recognizing adhesive epitopes on neutrophil CD11b reduced adhesion to HUVE by 47%, while mouse IgM had no effect. Incubation of glucose-grown *C. albicans* with saturating concentrations of purified human iC3b, but not equimolar concentrations of BSA, also inhibited candidal adhesion to HUVE [19].

Surface expression of candidal iC3b receptors was highest in clinical and laboratory isolates of *C. albicans*, followed by *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. lusitaniae*, *C. krusei*, and *S. cerevisiae* and correlated with adhesion to monolayers of HeLaS3 cells, an epithelioid carcinoma cell line [20].

Subsequent studies identified a role for RGD tripeptides in epithelial adhesion of *C. albicans* and *C. tropicalis*, the two most adherent yeast species. Incubation of *C. albicans* with anti- α M monoclonal antibodies, with purified iC3b (which contains an RGD sequence), or with 9-15mer peptides encompassing the RGD sequence in iC3b inhibited epithelial adhesion significantly [21]. Purified fibronectin or RGD peptides from the fibronectin sequence failed to block *C. albicans* adhesion to HeLa cells, but significantly blocked the adhesion of *C. tropicalis* [21].

Cloning of the INT1 Gene and Structure of the *Int1* Protein

The gene *INT1* was identified from a *C. albicans* DNA library composed of 3.0-3.8 kbp *EcoRI* fragments by an initial screen with a 314 bp *EcoRI/SmaI* cDNA fragment derived from the transmembrane domain of human α M [22]. Two hybridizing clones contained a 3.5 kbp *EcoRI* insert and failed to hybridize with degenerate oligonucleotides from the *S. cerevisiae* gene *USO1*. A 500 bp *HindIII* subfragment from one of these clones was used to screen 20,000 clones from a library of *C. albicans* 10261 genomic DNA. The largest hybridizing insert, a 10.5 kbp *SaII* fragment, was isolated by agarose gel electrophoresis, cloned, and sequenced. Analysis of the nucleotide sequence revealed an open reading frame sufficient to encode a 1661 residue polypeptide with a theoretical molecular mass of 187,989 Daltons and no extensive homologies with other proteins in the 1995 database [22]. BESTFIT sequence analysis located a putative I domain at amino acids 230-470 and three potential, partial MIDAS motifs for the coordination of divalent cations. There was an RGD sequence at residues 1149-1151, and a tyrosine residue at aa 1635, distal to a putative membrane-spanning region [22]. A pleckstrin homology domain at the C-terminus (aa 1529-1632) had no apparent function [23,24] [Figure 4].

Functions of *Int1*

Adhesion

Our first approach was to express *Int1* in the non-adherent yeast *Saccharomyces cerevisiae* [25]. We reasoned that *C. albicans* might have many adhesins (more than 20 now identified) and that a mutant deficient in just one might have no detectable loss of adhesion. *S. cerevisiae* transformed with vector alone was poorly adherent (<10%) to HeLa cells, while *S. cerevisiae* expressing *Int1* adhered almost as well as wild type *C. albicans* (30% vs. 42%). Disruption of both copies of *INT1* in *C. albicans* reduced adhesion, which could be blocked in both wild type *C. albicans* and in *S. cerevisiae* by antibodies against *Int1* [25].

Hyphal Morphogenesis

Surprisingly, *S. cerevisiae* expressing *INT1* grew in filamentous form, like *C. albicans* hyphae. Moreover, disruption of both copies of *INT1* in *C. albicans* completely inhibited filamentation on solid medium. Filamentous growth was restored by reintegration of one copy of *INT1* [25].

Virulence in a Murine Model

In a tail-vein injection model in outbred ICR mice, wild type *C. albicans* with two copies of *INT1* was associated with 0% survival, while the double disruptant ($\Delta int1$) permitted 90% survival. Mice injected with either the heterozygote (*INT1/int1*) or the reintegrant (*int1/int1*/

INT1) displayed intermediate survival (30-40%). Subsequently, *C. albicans* mutants disrupted for *INT1* exhibited decreased ability to colonize the murine intestinal tract [26,27].

Int1-Dependent Superantigen Effects

So why does the presence of *INT1* kill mice? Patients with candidemia exhibit many of the findings of systemic inflammatory response syndrome: sustained fever, hypotension, and elevated levels of pro-inflammatory cytokines such as TNF α and IFN γ [28-30]. We therefore sought an Int1-dependent toxin that elicits a potent inflammatory response.

Because the amino terminus of Int1 had 56% identity with the MHC Class II binding site of *Mycoplasma arthritidis* molecule (MAM), a well-characterized superantigen [31], initial studies examined the ability of wild type *C. albicans* (Int1⁺) and an *INT1* double disruptant (Int1⁻) to activate human T lymphocytes, expand particular V β subsets, and elicit pro-inflammatory cytokines.

In co-culture with PBMC's from five adult donors, the Int1⁺ strain was associated with up-regulation of the IL-2 receptor (CD25) in all donors; isogenic Int1⁻ mutants were inactive [32]. These effects were statistically significant on days 4-7 ($p < 0.02$). Two Int1⁺ strains and their respective reintegrants activated T lymphocytes, as measured by expression of CD25 and CD69. Int1⁻ isogenic strains were significantly less active ($p < 0.002$). Both CD4⁺ and CD8⁺ cells were activated, as were CD4⁺/CD8⁺ double positive cells; a similar pattern was noted with staphylococcal enterotoxin B, which served as a control superantigen. These effects required the presence of antigen-presenting cells and were not inhibited by paraformaldehyde.

All donors expanded V β subsets 2, 3 or 14 in Int1-dependent fashion [32]. One donor also expanded V β 7.1. Expansion of V β subsets by Int1⁻ cells was not observed. More than 75% of V β 2 and V β 14 T cells also expressed CD25, confirming that expansion correlated with activation. Both Int1⁺ and Int1⁻ cells elicited TNF α and IL-6; however, only Int1⁺ cells elicited IFN γ . IFN γ is therefore the Int1-dependent cytokine. Neither IL-4 nor IL-10 was detected in response to Int1⁺ or Int1⁻ *C. albicans*. MA b 163.5, a murine IgG1 raised to linear peptides spanning amino acids 239-278 of Int1, significantly inhibited T lymphocyte activation, expansion of V β subsets 2 and 14, and production of IFN γ [32].

When the first 263 amino acids of Int1 were expressed, this polypeptide (Pep₂₆₃) also activated T lymphocytes, expanded V β subsets 2 and 14, and elicited IFN γ . These effects required the presence of Int1⁻ blastospores and were significantly inhibited (50-70%) with 50 μ g/ml of MA b 163.5, but not by an equal concentration of an irrelevant murine IgG1. Neither mAb 163.5 nor murine IgG1 inhibited T cell activation or expansion of the V β 14 subset in response to staphylococcal enterotoxin B. Therefore, mAb 163.5 is specific for Int1 [32].

Native Pep₂₆₃ concludes with the sequence ₂₆₀KLKH₂₆₃. The full-length construct of 263 amino acids activated T lymphocytes and expanded V β subsets 2 and 14, but truncation constructs ending in K₂₆₂, L₂₆₁ or K₂₆₀ were no more active than medium alone. Substitution of A, D, or R for H₂₆₃ did not restore activation of T lymphocytes or expansion of V β subsets 2 and 14 [32]. These results accord with the observation that mutation of key histidine residues in classical superantigens aborts their potency [33-35].

When antigen-presenting cells were examined by the binding of mannose-BSA as a marker for the mannose receptor (MR), an antibody against HLA-DR bound to 90.3% of these cells; however in the presence of biotinylated Pep₂₆₃ the detection of HLA-DR was reduced more than 5-fold (from 90.3% to 15.9% of cells) [32]. Thus in the presence of Int1⁻ blastospores, binding of Pep₂₆₃ to antigen-presenting cells interferes with the detection of a class II antigen.

In the urine of a 43-day old premature infant with catheter-associated *C. albicans* fungemia, Western immunoblotting with mAb 163.5 detected immunoreactive bands at 44 and 22 kDa; bands of identical molecular weight were found when purified Pep₂₆₃ was incubated in donor urine but were not present in uninfected urine. These results show that Pep₂₆₃ can be generated *in vivo* [32].

Role of Heparin in Expression of the *C. albicans* Superantigen

The anti-coagulant heparin is commonly used to ensure patency of central venous catheters, which are identified as prime risk factors for candidemia in several clinical studies [36-38]. This clinical association led us to test whether heparin, in doses that one might use in an intravascular catheter, somehow augmented the expression of the candidal superantigen.

Our unpublished studies have shown that heparin does indeed trigger the exposure of Pep₂₆₃, as measured by binding of monoclonal antibody 163.5. Exposure of Pep₂₆₃ could be detected within 30 minutes. This phenomenon was reproduced with several other sulfated glycosaminoglycans [S.M. Kar, H Season, and M.K. Hostetter, unpublished data].

Binding of heparin, a strong anion, to proteins changes their conformation [39], and Int1 is readily accessible at the surface of the candidal cell wall. A search for heparin binding sequences (consensus sequence = BBXB, where B is any basic amino acid and X is any nonbasic/nonacidic amino acid) identified more than 15 at sites distal to Pep₂₆₃ [K.G. Tan and M.K. Hostetter, unpublished data]. Heparin affinity chromatography enabled the heparin binding site to be mapped to a four amino acid sequence. Mutation of this site abrogated binding of biotinylated heparin and exposure of Pep₂₆₃ [K.G. Tan and M.K. Hostetter, unpublished data].

Conclusions

Using biochemical and immunological techniques, we have identified a receptor for non-covalently bound iC3b in the cell wall of clinical and laboratory isolates of *C. albicans*. While initially demonstrated to play a role in RGD-mediated adhesion to epithelial cells and to inhibit phagocytosis, this protein, called Int1, is now known to contain a potent superantigen at its amino terminus (Pep₂₆₃). Pep₂₆₃ is exposed by heparin, is generated *in vivo*, and is inhibited by a monoclonal antibody, MAb 163.5.

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Figure 1.

Diagram of Opsonization (left panel), in which C3b is covalently deposited. Cleavage to iC3b exposes a binding site for attachment of neutrophil CR3, and phagocytosis ensues (center panel). Non-covalent binding of iC3b to *Candida albicans* perturbs CR3-mediated phagocytosis (right panel). Reprinted reference 9, with permission.

Figure 2. Histograms depicting binding of antibodies against complement receptors 1, 2, and 3 to *C. albicans* blastospores. Reprinted from reference 9, with permission.

Figure 3.

Saturable binding curves for the non-covalent binding of purified iC3b to the surface of *C. albicans* blastospores from blood isolates 296 (left) and 311 (right). Reprinted from reference 9, with permission.

Figure 4.

Diagram of putative and proven functional sites in the *C. albicans* cell wall protein Int1. Amino acids 1-263 comprise the superantigen Pep₂₆₃₋₂₃₉. MHC₂₅₄ is the region that shares homology with the MHC Class II binding site in the *Mycoplasma arthritidis* superantigen. D-H-N-S are putative MIDAS motifs. ₁₁₄₉RGD₁₁₅₁ is a site that mediates binding to epithelial cells.