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Identification of whole pathogenic cells by monoclonal antibodies generated against a specific peptide from an immunogenic cell wall protein

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ABSTRACT

We selected the immunogenic cell wall ß-(1,3)-glucosyltransferase Bgl2p from *Candida albicans* as a target protein for the production of antibodies. We identified a unique peptide sequence in the protein and generated monoclonal anti- *C. albicans* Bgl2p antibodies, which bound in particular to whole *C. albicans* cells.

1. Introduction

Pathogen detection and identification are major challenges for correct management of infectious diseases. Therefore, early and rapid identification of the infectious agent is of high importance for proper treatment of patients.

Culture based classical methods of clinical diagnostics are time consuming and may not be appropriate in the case of slow growing (Brodie and Schluger, 2005) or hardly cultivable microbes (Lindstrom and Korkeala, 2006).

Other procedures relying on the detection of nucleic acids (Ahmad et al., 2012; Pryce et al., 2003) show the required specificity, however, they require additional sample preparation steps such as cell disruption and nucleic acid amplification, thus delaying the rapid identification of the pathogen.

Indirect methods, which detect microbial carbohydrates (Lew et al., 1982; Ostrosky-Zeichner et al., 2005) or metabolites (Perl et al., 2011), rather than the whole pathogenic cell itself, often lack the desired specificity. Similarly, the detection of antibodies in sera of infected individuals may not be appropriate in the case of immunocompromised patients, as antibody production in those individuals is variable (Wahyuningsih et al., 2000).

Recently, the use of matrix-assisted laser desorption ionization time-of-flight mass-spectrometry (MALDI-TOF MS) analysis had been used for pathogen identification in blood cultures (Hoyos-Mallecot et al., 2014; Konnerth et al., 2014). However, the high costs of mass spectrometers prevent the wide application of MALDI-TOF MS based methods in clinical diagnosis.

Therefore, alternative approaches for the early, rapid, simple, cost effective and correct identification of pathogens are urgently needed.

The detection of whole microbial cells by antibodies is a well-established method, which evades many disadvantages of the aforementioned methods. However, frequently, antibodies are generated with the whole microorganism used as antigen. Thus, the specificity of the resulting antibodies is not well-defined and tedious selection procedures are required.

Using the most frequent human fungal pathogen *Candida albicans* as example, we aimed to develop monoclonal antibodies with pre-defined specificities, allowing the early and rapid identification of whole microbial cells. We made use of the nowadays available huge knowledge of genome sequences of various pathogens and of protein 3D-structures.

Abbreviations: CWPs, cell wall proteins; NACS, non-albicans *Candida* species; FACS, fluorescence automated cell sorting; GH, glycosylhydrolase.

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The antibodies should be designed to detect a specific epitope from a selected cell wall protein (CWP) of *C. albicans*. The target protein should be highly abundant in the cell wall and the epitope should be unique, also among other pathogens and should be present on the surface of the protein. This strategy should avoid subsequent selection of antibodies with suitable specificities to identify the pathogen in the presence of other microorganisms.

C. albicans is a commensal yeast colonizing 30–70% of healthy individuals (Gow et al., 2012). Patients in intensive care units of hospitals are at high risk to acquire systemic infections caused by *Candida* spp., due to immune system disorders caused by the major disease or the applied therapies. Together, *Candida* spp. constitute the fourth highest cause of nosocomial bloodstream infections with a lethality rate of up to 40% (Pfaller and Diekema, 2007).

The cell wall is the outermost compartment of fungal cells. It is a network of three major polysaccharides, to which CWPs are attached (Gozalbo et al., 2004). According to the nature of their attachment to the *C. albicans* cell wall, covalently and non-covalently bound CWPs can be distinguished. Non-covalently bound CWPs are entrapped in the polysaccharide layers. They can be removed from the cells by heat (Klebl and Tanner, 1989) or with denaturing agents or a combination of both (de Groot et al., 2004; Rico et al., 1997).

Any suitable target CWP should fulfill the following criteria:It should be:

(i) Accessible for antibody binding, i.e. it must be attached to the cell surface and exposed to the environment.
(ii) Abundant to allow sensitive detection.
(iii) Not present in humans and have epitopes with low homology to amino acid sequences of proteins from other pathogens or commensals in order to avoid cross-reaction.

Several studies have previously identified antigenic proteins of *C. albicans* which could evoke an immune response in the infected host (Gutierrez et al., 1993; Pitarch et al., 1999; van Deventer et al., 1994). Many of these proteins were however of cytosolic functions, such as glycolytic enzymes. While some of these proteins, such as Eno1p, Tdh3p, Adh1p or Tef1p, were reported to be also localized on the cell surface (Eroles et al., 1997; Gil-Navarro et al., 1997; Urban et al., 2003), their high conservation between humans and microorganisms (Martinez et al., 1998), deemed them to be poor candidates as diagnostic targets.

In recent studies, the ß-(1,3)-glucosyltransferase Bgl2p was identified as one of the strongest antigenic proteins of *C. albicans* (Clancy et al., 2008; Mochon et al., 2010; Pitarch et al., 2006). Bgl2p is highly abundant in the *C. albicans* cell wall (Angiolella et al., 2009) and lacks a homologue in humans (Chaffin, 2008). Homozygote Δ*bgl2* deletion mutants showed attenuated virulence in a murine model compared to an isogenic parental strain suggesting a role for this protein in virulence (Sarthy et al., 1997). Furthermore, interaction of Bgl2p with basic proline rich proteins (bPRP) of the human saliva was shown, suggesting that Bgl2p also functions as adhesin (Jeng et al., 2005).

For these reasons, we chose *C. albicans* Bgl2p as target protein for antibody generation. Unique peptides derived from the protein amino acid sequence should be used for immunization of mice. The peptides were to be selected via sequence comparisons among proteins from all organism classes. Significant differences should be present to the sequences of the same regions of Bgl2p orthologues from other *Candida* spp., in particular in the *Candida glabrata* Bgl2p orthologue. Distinguishing between *C. albicans* and *C. glabrata* is of high clinical importance as *C. glabrata* shows increased resistance to antimycotic substances to which *C. albicans* is susceptible (Fidel et al., 1999; Kragelund et al., 2013). The localization of selected peptide sequences in the Bgl2p 3D-structure was to be estimated from a structure model.

Antibodies tested positive for binding the selected peptides were analyzed for binding recombinant Bgl2p and whole *C. albicans* and *C. glabrata* cells. The specificity was also evaluated with the Δ*bgl2* deletion mutant. Analysis was done by fluorescence immunoanalysis and flow cytometry, the latter allowing the analysis of even single cells. In particular, antibodies released by the hybridoma cell clone C25HD4A8 directed against the Bgl2a10 peptide had most of the desired properties. Thus the validity of our strategy was confirmed.

2. Materials and methods

2.1. Microbial strains and culture conditions

SC5314 (Gillum et al., 1984) and ATCC2001 (Csank and Haynes, 2000) were used as *C. albicans* and *C. glabrata* wild-type strains respectively. FB63-1 and FB63-3 (*ura3Δ::imm434/ura3Δ::imm434*, *iro1/iro1*, *arg4::hisG/arg4::hisG*, *his1::hisG/his1::hisG*, *bgl2::ARG4/bgl2::URA3*, Fungal Genetic Stock Center (FGSC), Kansas, USA) (Nobile and Mitchell, 2009) were the Δ*bgl2* deletion mutants, while DAY286 (*ura3Δ::imm434/ura3Δ::imm434*, *iro1/iro1*, *ARG4::URA3::arg4::hisG/arg4::hisG*, *his1::hisG/his1::hisG*, also obtained from FGSC) (Davis et al., 2002) served as marker matched reference stain. *Escherichia coli* strains were XL-1 BLUE (Arias et al., 2000) and Rosetta™ 2(DE3) (Machner and Isberg, 2006).

All yeast strains were cultivated overnight (16–24 h) from frozen glycerol stocks in YPD medium (Sigma-Aldrich Y1375) at 30 °C (Multitron Shakers, Infors-HT, 160 rpm). Growth was followed by measurements of optical densities of cultures at $\lambda = 600$ nm (OD_{600}) in transparent 96 well plates by the μQuant microtiterplate reader (Biotek, Bad Friedrichshall, Germany) in triplicates (each 180 μL). Preparation of all cultures was performed under sterile conditions.

2.2. Bgl2p extraction

An overnight culture of *C. albicans* in YPD was diluted in YPD to an OD_{600} of ~0.1 and cultivated at 30 °C for another 16–18 h. For extraction of Bgl2p from *C. albicans* at different growth stages, 20 ml of YPD medium was inoculated with cells from a glycerol stock of DAY 286 so that after 18 h an OD_{600} of ca. 0.4 was reached. This culture was split into three aliquots, each diluted in YPD to an OD_{600} of ca. 0.1 and grown at 30 °C until ODs_{600} of ca. 0.4 (early exponential phase), 1.5 (late exponential phase) and 3.2 (stationary phase), respectively, were reached.

For all Bgl2p extraction experiments, cells were harvested by centrifugation [4500 ×*g*, 5 min, room temperature (RT)] and washed with PBS, pH 7.4 (8.0 g · L^{-1} NaCl, 0.2 g·L^{-1} KCl, 1.44 g·L^{-1} $Na_2HPO_4 \cdot 2 \cdot H_2O$, 0.24 g·$L^{-1}$ KH_2PO_4). The supernatant was removed and 30–100 μL PBS was added to the cell pellet to obtain a cell suspension. Proteins were extracted by boiling these cells at 90 °C for 10 min. The suspension was centrifuged (10000×*g*, 4 °C, 10 min) and the supernatant was collected. Centrifugation was occasionally repeated once to remove residual cells. The obtained supernatant was analyzed by SDS-PAGE for the presence of Bgl2p (Suppl. methods 1).

2.3. Design, expression and purification of recombinant Bgl2p

A *C. albicans* Bgl2p (*Ca*Bgl2p) encoding gene was designed with restriction sites altered by silent nucleotide substitutions to facilitate cloning. Coding sequences for 3× FLAG- and 8× His-Tags were added at the 3′ end followed by a stop codon. A *NcoI*-digestion site was included after the coding sequence of an 18 aa signal peptide (Angiolella et al, 2009) which allows cloning of two versions of the gene, one encoding the full length Bgl2p (rBgl2p, Suppl. Fig. 1A, Suppl. methods 2), while the other encodes Bgl2p lacking the signal peptide (rBgl2p-SP). The resulting amino acid sequence was