Rev Iberoam Micol. 2012;29(3):172–174

Revista Iberoamericana de Micología

www.elsevier.es/reviberoammicol

Note

Phosphoglycerate kinase and fructose bisphosphate aldolase of *Candida albicans* as new antigens recognized by human salivary IgA

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ARTICLE INFO

Article history:
Received 28 February 2011
Accepted 27 July 2011
Available online 11 August 2011

The present work is dedicated to Dr. José Pontón, a great friend and better researcher, who passed away in July 2010.

Keywords:
Candida albicans
Antigens
Immunoglobulin A
Phosphoglycerate kinase
Fructose bisphosphate aldolase

ABSTRACT

Background: *Candida albicans* is an opportunistic dimorphic fungus commonly present in the human oral cavity that causes infections in immunocompromised patients. The antigen variability, influenced by growth conditions, is a pathogenicity factor.
Aims: To determine the effect of nutritional and heat stress on the antigen expression of *C. albicans*, and to identify major antigens recognized by human salivary secretory immunoglobulin A (sIgA).
Methods: Under various different nutritional conditions, heat shock was induced in *C. albicans* cells in stationary and exponential growth phases. The expression of protein determinants of *C. albicans* was assessed by Western blot analysis against human saliva. The antigens were purified and characterized by two-dimensional electrophoresis and identified by protein microsequencing.
Results: Five antigens recognized by salivary IgA were characterized as mannoproteins due to their reactivity with concanavalin A. They did not show reactivity with anti-heat shock protein monoclonal antibodies. Two of them (42 and 36 kDa) were found to be regulated by heat shock and by nutritional stress and they were identified as phosphoglycerate kinase and fructose bisphosphate aldolase, respectively.
Conclusions: These glycolytic enzymes are major antigens of *C. albicans*, and their differential expression and recognition by the mucosal immune response system could be involved in protection against oral infection.

Fosfoglicerato quinasa y fructosa bifosfato aldolasa de *Candida albicans* como nuevos antígenos reconocidos por la IgA salival humana

Palabras clave:
Candida albicans
Antígenos
Inmunoglobulina A
Fosfoglicerato quinasa
Fructosa bifosfato aldolasa

RESUMEN

Fundamento: *Candida albicans* es un hongo dimórfico oportunista que, con frecuencia, está presente en la cavidad oral del ser humano donde da lugar a infecciones en pacientes inmunocomprometidos. Influida por las condiciones de crecimiento, la variabilidad antigénica es un factor de patogenicidad.
Objetivos: Determinar el efecto del estrés nutricional y térmica en la expresión antigénica de *C. albicans*, e identificar los principales antígenos reconocidos por la inmunoglobulina secretora A (sIgA) salival humana.
Métodos: En diferentes condiciones nutricionales, se indujo un choque térmico en células de *C. albicans* en fase de crecimiento estacionario y exponencial. La expresión de los determinantes proteicos de *C. albicans* se analizó mediante inmunotransferencia frente a la saliva humana. Los antígenos se purificaron y caracterizaron mediante electroforesis bidimensional y se identificaron mediante microsecuenciación de proteínas.
Resultados: Se caracterizaron cinco antígenos reconocidos por la IgA salival como manoproteínas debido a su reactividad con la concanavalina A. Ninguno manifestó reactividad con los anticuerpos monoclonales

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1130-1406/$ – see front matter
doi:10.1016/j.riam.2011.07.004

anti-proteína de choque térmico. Se encontró que el choque térmico y el estrés nutricional regulaban dos de ellos (de 42 y 36 kDa) identificados como fosfoglicerato quinasa y fructosa bifosfato aldolasa, respectivamente.

Conclusión: Estas enzimas glucolíticas son antígenos mayores de *C. albicans*, y su expresión diferencial y el reconocimiento por el sistema de la respuesta inmunitaria de la mucosa podrían participar en la protección frente a las infecciones orales.

Candida albicans is an oral commensal yeast in around a third to a half of the healthy population.[8] However, this fungus is the main cause of superficial forms of candidiasis, and clinical observations indicate that mucocutaneous *Candida* infections are commonly associated with a defective immune response.[16]

Saliva is an important element of the protective barrier provided by the oral epithelium[1] because it contains a variety of proteins, in particular secretory immunoglobulin A (sIgA), which affords protection by inhibiting adherence and penetration into mucosal tissues of microorganisms,[14] including *C. albicans*.[19] The sIgA coating of *Candida* cell walls is observed in clinical smears from patients with oral and vaginal candidiasis. Some studies[13,15] have identified antigens of *C. albicans* that react with salivary sIgA, such as heat shock mannoproteins. They are expressed at higher levels in cells grown at 37 °C than in cells grown at 24 °C. In this work, we have identified two glycolytic enzymes regulated by heat and nutritional stress as new antigens recognized by antibodies in human saliva.

Materials and methods

Microorganisms and culture conditions

Three *C. albicans* strains, UPV1360, VW32 and UPV1413, were used. The strains were grown in Sabouraud Dextrose Broth (SDB) with 4% of glucose at 24 °C and stirred at 120 rpm, for 9 h or 24 h to obtain exponential phase or stationary phase cells, respectively. They were harvested by centrifugation (1250 × *g*, 5 min), washed twice with sterile 50 mM phosphate-buffered saline (PBS, pH 7.4), and then resuspended in one of the following culture media: (i) SDB in which the cells had been growing; (ii) fresh SDB; (iii) fresh SDB without peptone; or (iv) fresh SDB without glucose. All of them were incubated at 37 °C for 15, 30, 60 and 120 min to study the heat shock response, or at 24 °C for 120 min as the control.

Saliva collection

Whole saliva from 13 *Candida*-free healthy individuals (7 men and 6 women; 20–40 years old) was collected with Salivettes (Sarstedt, Barcelona, Spain), after they had signed an informed consent form. Half of the sample volume was pooled and the other half was stored individually. After centrifugation, as described above, supernatants were adsorbed with *C. albicans* UPV1360 cells grown at 24 °C for 24 h to eliminate non-specific binding in the Western blot (WB).[5]

Electrophoresis and Western blot analysis of antigen extracts

The antigen extracts were obtained from 10^{10} *C. albicans* cells and analyzed by one-dimensional electrophoresis (1-DE) according to the method described by Calcedo et al.[5] The two-dimensional electrophoresis (2-DE) was carried out as in Hernando et al.[7]

The 1-DE and 2-DE gels were transferred to PVDF membranes (Immobilon-P; Millipore, Billerica, MA, USA) and mannoproteins were detected using concanavalin A.[7] For WB analysis, adsorbed saliva or anti-Hsp 90 (Bionova, Madrid, Spain), 70, 60, and 25 kDa (Sigma, St. Louis, MO, USA) mouse monoclonal antibodies were used as primary antibodies, and goat anti-human IgA and anti-mouse IgG peroxidase (Sigma) as secondary antibodies. Bands were revealed with diaminobenzidine (DAB)[5] and analyzed using the Bio Image 50S system with the Whole Band and 2D Analyzer software (Millipore).

Protein purification and microsequencing

Crude extract (10 mg) was fractionated using the 491 Prep Cell and the Econo System (Bio-Rad, Hercules, CA, USA) in a 28 mm diameter, 13% acrylamide/bis-acrylamide gel, at 1 ml/min following the manufacturer instructions. Fractions containing the 36 kDa or the 42 kDa band were recovered, dialysed, concentrated using 10 kDa Ultrafree-Biomax membranes (Millipore), and analyzed by 2-DE and WB. The spots were excised from the 2-DE gels and sequenced by Edman degradation (Proteomics Laboratory of the CNB, Cantoblanco, Madrid), and sequences obtained were identified by searching, either in SWISS-PROT, in TrEMBL or using NCBI's Basic Local Alignment Search Tool (BLAST).

Results and discussion

Of all the different types of infection caused by *Candida*, oral and vaginal candidiasis are the most common clinical presentations,[6,12] and the lack of sIgA against *Candida* could explain the recurrence of candidiasis in some patients.[18] Several HSPs of 205, 180, 140 and 110 kDa recognized by sIgA have already been identified by our group.[5,13,15] In this work, we have found five major antigenic components, 42, 36, 33, 27 and 25 kDa (Fig. 1), recognized by salivary sIgA. Two of them, 42 and 36 kDa, modified their expression in all the strains under different growth and stress conditions, and commercial anti-HSPs monoclonal antibodies did not react with them, suggesting that they could be fragments that do not contain the recognized epitopes or new HSP. These antigens might be expressed on *C. albicans* cells colonizing human mucosal surfaces, since in 76.9% and 69.2% of saliva samples there was a reaction with these antigens, respectively (data not shown), although we cannot rule out previous asymptomatic candidiasis in these healthy individuals. Only one of the saliva samples did not react with any of these antigens. The 42 kDa and 36 kDa antigens were identified as phosphoglycerate kinase (PGK) and fructose bisphosphate aldolase (FBA), respectively (Table 1). Both these antigens are mannoproteins involved in glycolytic metabolism, and therefore their expression is coregulated by nutritional and heat stress.

PGK, in optimal nutritional conditions (Fig. 1a), is an immunodominant antigen, but it was not expressed in the stationary growth phase (Fig. 1c) or under conditions of nutritional stress (Fig. 1g and h). However, it was expressed again in stationary phase cells and under starvation conditions after a heat shock (Fig. 1f and h). So, PGK expression seems to need an active metabolic phase or heat shock treatment. This antigen, which has been found in the *C. albicans* cell wall,[2] is an enzyme involved in ATP production,[21]

Table 1
Protein identification after sequencing. Experimental and theoretical molecular mass (Mr) and isoelectric point (pI).

Protein	Entry no.[a]	Experimental Mr	Theoretical Mr	Experimental pI	Theoretical pI
PGK	P46273	42–46 kDa	45.179 kDa	5.5–5.8	6.07
FBA	Q9URB4	36–40 kDa	39.215 kDa	6.0–6.3	5.7

[a]Entry number according to the UniProtKB database.

and could generate energy for local cell wall biosynthesis as well as for cytoplasmic activity.[4] Reactive oxygen species are involved in the transcriptional activation of the *PGK1*gene. In fact, it has been recently described as a potential protein biomarker of intracellular oxidative status in human colon carcinoma cells.[9] In the stationary growth phase, the overexpression of PGK we detected could be related to higher requirements for energy by enhancing ATP production.

In contrast, FBA expression was observed in the stationary phase of growth and under starvation conditions. *C. albicans* FBA1p belongs to the family of class II aldolases found predominantly in fungi and prokaryotes,[10] and it is involved in the formation of the cell wall.[3] Further, antibodies against FBA have been detected in serum samples from human, monkeys and mice infected by *C. albicans*, protozoa or parasitic worms.[11] Recently, it was shown that a vaccine combining β-mannan and the FBA peptide conferred immunity against candidiasis.[20] On the other hand, considering that *FBA1* is a single gene in *C. albicans* and that it exhibits strong sequence similarity to its orthologues in *Schizosaccharomyces pombe*, *Aspergillus nidulans* and *Neurospora crassa*, an antifungal agent directed against *C. albicans* FBA1 might have broad specificity.[17]

In conclusion, at least one of the two glycolytic enzymes of *C. albicans*, phosphoglycerate kinase and fructose bisphosphate aldolase, whose expression is regulated by nutritional and heat stress, were recognized as immunogenic in the most of the studied human saliva (92.3%) samples. In spite of the fact that continuous contact with *C. albicans* cells or cross-reactivity with other pathogens might be the origin of these specific sIgAs in healthy individuals, specific sIgAs against these antigens could be useful for controlling *Candida* growth and, hence, the infection process. In particular, these antigens could be of interest in antifungal therapy and explain, at least in part, the pathogenesis of mucosal *C. albicans* infection.

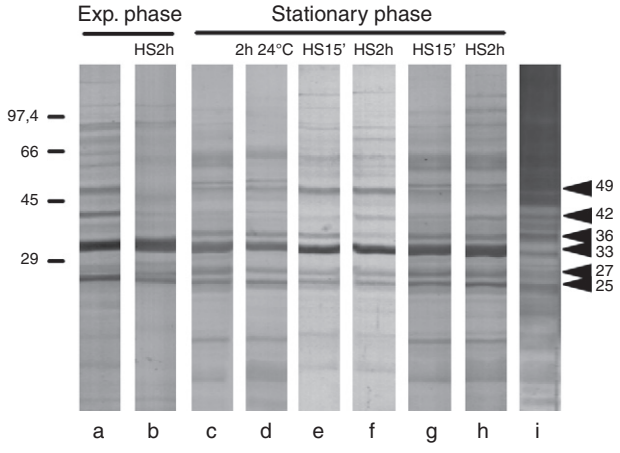

Fig. 1. Western blot of protein extracts from: (a) exponential phase cells; (b) exponential phase cells incubated for 2 h at 37 °C in the SDB in which cells had been growing; (c) stationary phase cells; (d) stationary phase cells incubated for 2 h at 24 °C in fresh SDB; (e and f) stationary phase cells incubated for 15 min or 2 h at 37 °C in fresh SDB, respectively; (g and h) stationary phase cells incubated for 15 min or 2 h at 37 °C in SDB without peptone, respectively; and (i) mannoprotein detection using concanavalin A in stationary phase cells incubated for 2 h at 37 °C in fresh SDB.

Acknowledgements

This work has been partially supported by a SAIOTEK Grant (S-PE09UN41), a Consolidated Research Group IT343/10 of the Government of the Basque Country, and a grant from the University of the Basque Country (UE 08/14).

We thank Ideas Need Communicating Language Services SL for proofreading the English text.

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