Differences in the *Candida albicans* antigenic expression after heat shock and infection

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Summary

Heat-shock and infection induce changes in protein expression in *C. albicans*. To investigate if these alterations induce changes in antigenicity, we have compared the reactivity mediated by IgA antibodies of protein extracts from a strain of *C. albicans* and the same strain recovered from an infected animal, both at 24°C and 37°C. The antigenic variability was detected mainly in antigens recognized by salivary IgA. Antigens of 223, 205, 180 and 140 kDa were over-expressed in both strains at 37°C, indicating that variations due to heat shock were present before and after infection. The antigens were characterized as mannoproteins located at the outer side of the cell wall. An antigen of 61 kDa was also detected in which the expression decreased significantly after infection This was independent of heat shock.

Key words *Candida albicans*, HSP, Infection, IgA

Diferencias en la expresión antigénica de *Candida albicans* tras choque térmico e infección

Resumen

El choque térmico y la infección inducen cambios en la expresión proteínica de *C. albicans*. Para saber si estos cambios están relacionados con la antigenicidad, hemos comparado la reactividad mediada por anticuerpos IgA de un extracto proteico de una cepa de *C. albicans* y de la misma cepa recuperada de animales infectados, a las que se sometió a un choque térmico a 24°C y 37°C. La variabilidad antigénica afectó principalmente a los antígenos reconocidos por la IgA de la saliva. Antígenos de 223, 205, 180 y 140 kDa se sobreexpresaron en ambas cepas a 37°C, indicando que las variaciones debidas al choque térmico se producen antes y después de la infección. Los antígenos se caracterizaron como manoproteínas situadas en la parte externa de la pared celular.
Se detectó también un antígeno de 61 kDa cuya expresión fue independiente del choque térmico pero disminuyó significativamente después de la infección.

Palabras clave *Candida albicans*, HSP, Infección, IgA

The opportunistic pathogen *Candida albicans* is able to induce severe infections and death in animals and humans though it can be found on the skin surface and mucous membranes of many healthy mammals. This fungus is able to grow in three different morphologies, yeast, pseudohyphae and hyphae, depending on the growth conditions [1], this characteristic being considered a virulence factor. *In vivo*, the hyphal form is present only during invasive colonisation although both forms are found in infected tissues. The cell wall is the structure responsible for the characteristic shape in both morphologies. It acts as a permeability barrier and, due to its external position, is also the site of the initial interaction between the organism and the environment [2]. The interconnections between cell wall components, chitin and β-glucans as structural components and mannoproteins as matrix components, result in an intricate structure that is responsible for the chemical and mechanical stability of the fungal walls [3].

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The *in vitro* induction of germ tubes depends not only on the presence of certain nitrogen-containing nutrients, but also on the temperature shift from 24°C to 37°C or other stress conditions such as ethanol treatment [4]. The shift in temperature induces heat shock protein (HSP) synthesis. These HSPs are, in some cases, highly antigenic and contain highly conserved epitopes. It has been suggested that HSPs might be involved in some aspects of growth and differentiation of cells [5-8]. Antibodies to HSPs may cross-react with human HSPs causing immunologically mediated skin damage such as chronic mucocutaneous candidiasis (CMC) and rheumatoid arthritis [9]. Since *C. albicans* is a common commensal of human mucous membranes, individuals have developed various primary defence barriers. Among these host factors, saliva [10,11] and its major immunoglobulin, secretory IgA (sIgA), present in all mucous secretions [11], seem to play an important role in the control of oral candidiasis by inhibiting *C. albicans* adherence to host cells [12]. Actually, the significantly low IgA concentration observed in saliva from patients with AIDS has been related to the high incidence of oral candidiasis in these patients [13].

Recently, we have identified antigens of *C. albicans* reacting with salivary IgA as heat shock mannoproteins (HSMP) [14,15]. In this study we have investigated and characterised the expression of antigenic determinants of *C. albicans* reacting with IgA *in vitro*, under two different temperature conditions, 24 and 37°C, and after infection.

MATERIAL AND METHODS

Microorganism and culture conditions. For all studies, two *C. albicans* strains were used. *C. albicans* UPV 1360, originally isolated from a patient with vulvovaginal candidiasis, was maintained on Sabouraud-Dextrose agar by frequent transfer, and *C. albicans* UPV RSI that was isolated from mice infected with *C. albicans* UPV 1360 as follows. Mice were infected intravenously with 1 x 10^5 blastoconidia of *C. albicans* UPV 1360, three times during three successive weeks, and sacrified 24h later. Livers were removed, homogenated and plated on Sabouraud-dextrose agar containing chloramphenicol (0.5 g/l), to obtain isolated colonies.

Sampling of saliva and treatment. Saliva was obtained from healthy donors with Salivettes (Sarstedt) and adsorbed with blastoconidia of *C. albicans* UPV 1360 grown at 24°C during 24 h. Briefly, 2x10^9 *C. albicans* cells/ml in PBS were mixed with the same volume of saliva containing 0.01% PMSF. After 2 h at room temperature, the suspension was centrifuged at 1250 g for 5 min and the supernatant was collected and frozen at -20°C until used. When a double adsorption (DA) was required, saliva previously adsorbed with *C. albicans* UPV 1360 cells, was adsorbed again as above, except that *C. albicans* UPV RSI cells, heat shocked at 37°C, were used the second time.

Heat shock treatment . C. albicans cells blastospores grown in liquid Sabouraud at 24°C, 120 rpm, for 24 h were collected by centrifugation, washed twice with sterile PBS pH 7.4, and incubated in fresh Sabouraud previously heated at 37°C or 24°C, at 120 rpm for 30 min where the blastospore morpholgy was dominant.

Antigen extraction. C. albicans cells, either UPV 1360 or UPV RSI, were extracted with alkali under reducing conditions (AERC) [16] prior to and after heat shock. The cells (1010) were treated with 22 ml of 1.25 M NaOH and 5% β-mercaptoethanol for 15 min in ice. The proteins extracted were then precipitated by the addition of an equal volume of 50% (v:v) trichloroacetic acid. After 15 min, the samples were centrifuged and washed three times in 1M Tris. Cells and precipitated proteins were boiled for 5 min in 0.5 ml of SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% sucrose, 10% β-mercaptoethanol). Insoluble cellular material was removed by centrifugation at 10,000 g for 5 min. Supernatants containing the antigenic mixture were stored at -20°C. Protein concentration was determined using the BCA Protein Assay Reagent (Pierce).

SDS-PAGE and blotting. The antigenic mixtures were separated by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) according to the method described by Laemmli [17] in a minigel system, Mini-Protean II (Bio-Rad Laboratories). Electrophoresis was carried out in 8% or 12% slab gels at 200 V and 70 mA for 1h. The 8% slabs were used to separate the high molecular weight (HMW) proteins and the 12% slabs were used to separate intermediate and low molecular weight (LMW) proteins. The total amount of protein loaded was 140 µg per lane in 8% slabs and 40 µg per lane in 12% slabs. Low and High Molecular Weight Markers (Bio-Rad) were used as standards. After electrophoresis, the gels were blotted onto Immobilon-P membranes (Millipore) in LKB-NovaBlot Semy Dry System (Pharmacia) for 30 minutes at 4 mA/cm. After transfer, the Immobilon-P membranes were stained with Ponceau Red (Sigma).

Chemical characterization. Differential labelling of glycosylated proteins blotted onto Immobilon-P membranes was performed by the DIG (Digoxigenin) Glycan/Protein Double Labelling method (Boehringer Mannheim). In brief, the Immobilon membrane was washed in 50mM PBS (pH 6.5), incubated 20 min in 100 mM sodium acetate buffer pH 5.5, and washed in the same PBS three times for 5 min each. After incubation with 2 ml DIG hydrazide in 10 ml of 100 mM sodium acetate buffer pH 5.5 for 1 h at room temperature, the membrane was washed, incubated in the blocking solution and washed again as before. Finally, the membrane was incubated with 20 ml of anti-digoxigenin-HRP (Horseradish peroxidase) in 10 ml TBS (0,5 % v/v Tween 20 in 50mM PBS) for 1h, washed in the same buffer and incubated in the staining solution (10 ml Tris buffer pH 8.8, 50 ml TETON and 6 ml H_2O_2) for 20 min.

Mannoproteins were detected with Concanavalin A (ConA). First, the membrane was blocked with 10% BSA and washed three times with buffer A (1 mM $MgCl_2$, $MnCl_2$ and $CaCl_2$ in TBS). Then it was incubated 1h at 37°C with 10 mg/ml of ConA-HRP, washed with TBS and revealed with Diaminobenzidine (DAB).

Immunodetection of antigens. Membranes were blocked in 5% skim milk powder (OXOID) overnight at 10°C and then incubated with the adsorbed saliva diluted 1:2 in PBS-T (0,05% Tween 20 in PBS). Immunoreactive bands were revealed with DAB after binding with goat anti-human IgA or IgG peroxidase conjugate (Sigma) 1/100 in PBS. In order to assess whether IgA derived from the saliva of healthy individuals was specific for carbohydrates or protein antigenic determinants, Immobilon-P strips were previously treated with periodate according to the method described by Polonelli *et al.* [14].

RESULTS

In order to separate accurately high and low molecular weight heat shock antigens (HSA), two different polyacrylamide gels, 8% and 12%, were run, blotted and immunodetected with IgA. The rate between antigenic

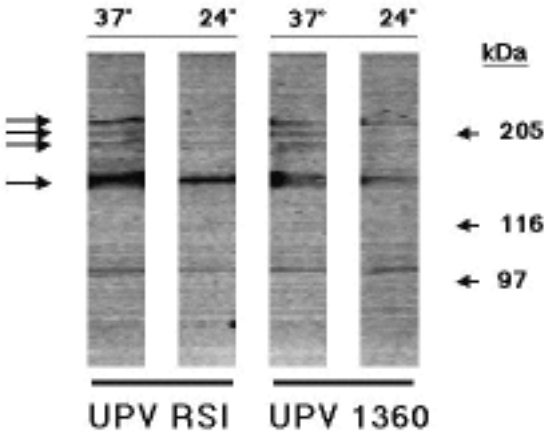

Figure 1. Immunoblot analysis with IgA of 8% polyacrylamide slabs gels loaded with *C. albicans* protein extract from cells grown at 24°C and heat shocked at 37°C. Two first lanes shown the reactivity of UPV RSI recovered from infection. The other lines are extracts from UPV 1360. The left arrows indicate antigens described and the arrowheads indicate the molecular weight standards.

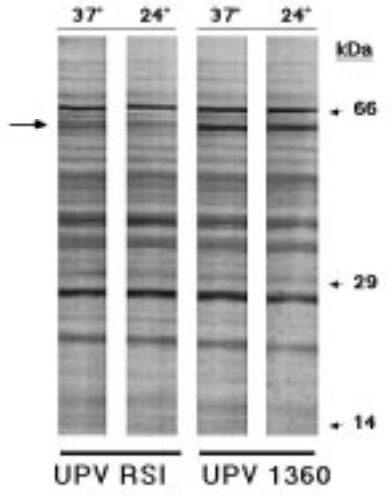

Figure 2. Immunoblot analysis with IgA of 12% polyacrylamide slabs gels loaded with *C. albicans* protein extract from cells grown at 24°C or those heat shocked at 37°C. Lines and arrows are indicated as in Figure1.

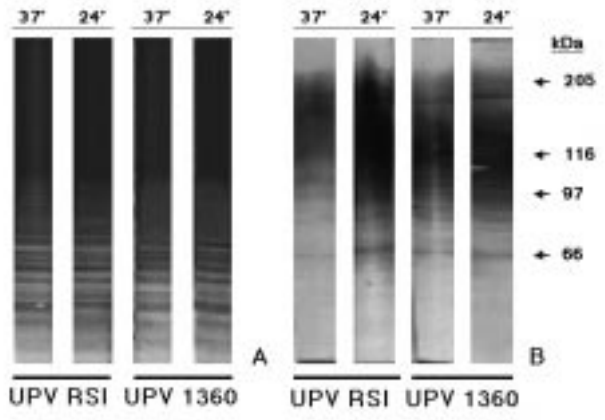

Figure 3. Glycoprotein detection by ConA lectin (A) and DIG glycan/protein method (B). 8% polyacrylamide slabs gels loaded with *C. albicans* protein extract from cells grown at 24°C or heat shocked at 37°C. Lines and arrows are indicated as in Figure 1.

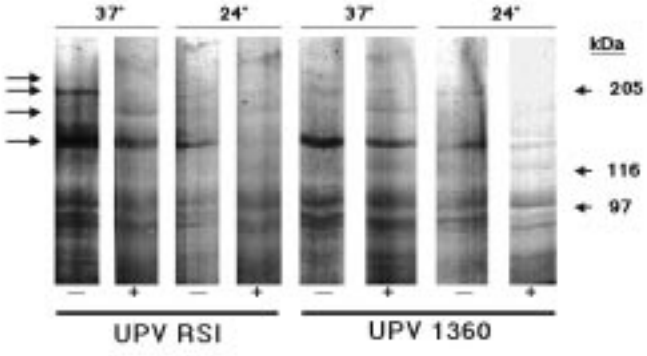

Figure 4. Immunoblot analysis with IgA of 8% polyacrylamide slabs gels loaded with *C. albicans* protein extracted from cells grown at 24°C or heat shocked at 37°C. Lanes oxidated with Metaperiodate (+) and lanes without treatment (-). The left arrows indicate antigens described previously and the arrowheads indicate the molecular weight standards.

protein and antibody happens to be critical to discriminate HMW antigens, being necessary to rise the antigenic protein amount up to 140 mg per lane.

Four HMW antigens (223, 205, 180 and 140 kDa) were over-expressed at 37° C, in cell wall extracts from both, UPV 1360 and UPV RSI (Figure 1). No intermediate or low molecular weight antigens were over-expressed at this temperature in any of the extracts compared to 24°C (Figure 2). Major antigens of, 21, 27, 34, 38, 47, 61 & 66 kDa, were found in both extracts, but the immunodominant antigen of 61 kDa, strongly expressed in the UPV 1360 extract, was much lower in the UPV RSI extract.

In order to characterize the HSAs, two previously described methods were used on blots from 8% gels: a) treatment with ConA-HRP to detect α-Man residues and b) differential labelling of glycosylated proteins. In the high molecular weight region an intense reaction was observed with both methods (Figure 3), indicating the glycoprotein and more specifically mannoprotein nature of the antigens (Figure 3 B). The 66 kDa band was also revealed as a glycoprotein.

To elucidate the nature of the epitopes involved in the antigenic reaction the immobilon membrane was oxidized with sodium metaperiodate and incubated with saliva. The 223 and 205 kDa HSAs were not recognised after oxidation, in any of the extracts, showing the glycosidic nature of the epitopes (Figure 4). There was also a decrease in the reactivity of the immunodominant 140 kDa HSA at 37°C, but the reaction could be clearly observed after oxidation indicating that the epitopes are both glycosidic and protein. Only the glycosidic moiety was expressed at 24°C. An increase in reactivity with the 180 kDa HSA at 37°C after oxidation was observed in both types of extracts, indicating that the antibodies in this case are specifically directed against the protein moiety.

The characterization of low molecular weight antigens after mild oxidation with metaperiodate is shown in figure 5. All of the antigens were recognized by the IgA of saliva before and after the heat shock. Mild oxidation showed the protein nature of the epitopes in both strains, including the 61 kDa antigen expressed in UPV 1360 extract.

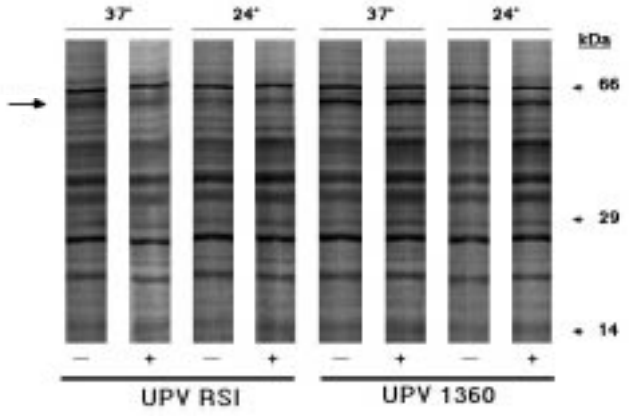

Figure 5. Immunoblot analysis with IgA of 12% polyacrylamide slabs gels loaded with *C. albicans* protein extract from cells grown at 24°C or heat shocked at 37°C. Lines and arrows are indicated as in Figure 4.

The localization of HSAs was determined by a double adsorption of saliva (Figure 6, DA lanes). Only the140 kDa antigen was recognized by IgA after adsorption, indicating that its expression was higher in the inner side of the cell wall, while the other HSAs are accesible to antibodies in the outer side of the cell wall.

No HMW antigen was recognized when anti IgG-HRP conjugate was used as the secondary antibody (Figure 6, IgG). With respect to LMW antigens (Figure 7), anti IgG conjugate identified bands of 66, 61, 47, 38 & 31 kDa from cells grown at both, 37°C and 24°C, showing a weak cross reactivity with IgA. However the general reactivity was lower with anti IgG than the reactivity exhibited against anti IgA.

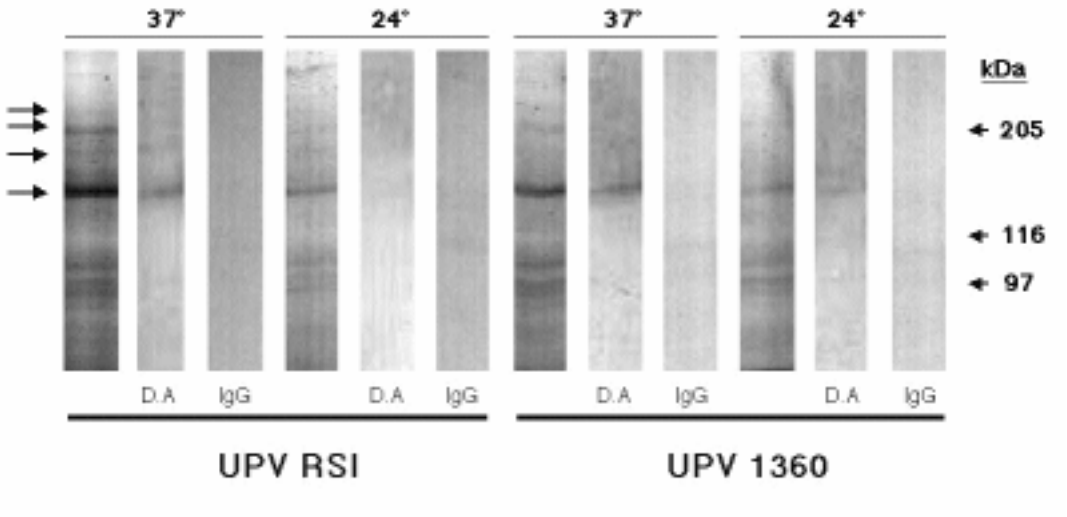

Figure 6. Immunoblot analysis with IgA and IgG of 8% polyacrilamide slabs gels loaded with *C. albicans* protein extract from cells grown at 24°C or heat shocked at 37°C. Double adsorption samples are indicated as (D.A) and antigens reacting with IgG conjugate are marked as IgG. The left arrows indicate antigens described and arrowhead indicate the molecular weight standards.

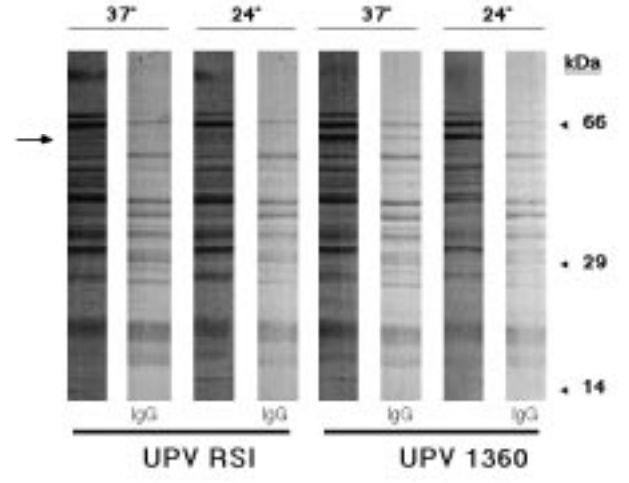

Figure 7. Immunoblot analysis with IgA and IgG of 12% polyacrylamide slabs gels loaded with *C. albicans* protein extract from cells grown at 24°C or heat shocked at 37°C. Arrows are indicated as in Figure 6.

DISCUSSION

Oral, invasive and vaginal candidiasis are the most frequent clinical presentations [14,18] of candidiasis. Antibodies to *C. albicans* present in both vaginal and oral secretions belong to the IgA and IgG classes of immunoglobulins. Inhibition of the adhesion of *C. albicans* to the surface of epithelial cells has been proposed as a possible role for IgA[19], consequently, low levels of IgA, the major immunoglobulin in mucous membranes [20] could explain the recurrence of candidiasis in some patients [21].

In this study we have analysed antigen expression in two *C. albicans* strains from different infections to determine if the effect produced by temperature, an important factor for germination and pathogenicity [1], is related to antigen expression after invasive infection. In previous studies, we have identified several HSPs recognized by sIgA [14,15]. In this study we have characterized some new HSAs (Figure 1), with apparent molecular mas-

ses of 223, 205, 180 and 140 kDa. The detection of these antigens was only possible when a high amount of protein was loaded in the gels, probably because their expression was quantitatively low. Because of this, they were only immunodetected by means of partial adsorption and dialysis of saliva to remove background due to nonspecific binding of antibodies.

We have shown (Figure 3) the glycosidic nature of the HMW proteins, particularly rich in α-Man residues (Figure 3A) that are characteristic components of *C. albicans* cell wall polysaccharide [22]. ConA showed a more specific and sensitive reaction than DIG glycan/ protein double labelling method (Figure 3B). After a mild periodate oxidation (Figure 4), the glycosidic nature of the epitopes recognized by IgA in the 223 and 205 kDa HSA has been demonstrated. The 180 kDa antigen was more readily observed after oxidation, since most likely the glycosidic moiety of the antigen could hide this epitope, but after oxidation the IgA antibodies could react with the proteinaceous epitope. An antigen with the same MW has been described [23, 24] as a protein with adhesion ability localized to the cell wall where the germinal tube originates.

The 140 kDa antigen was recognized at 24°C as a glycoprotein with a glycosidic epitope (Figure 4), but after heat shock, a protein epitope was also recognized indicating a newly synthesized protein epitope. Both strains expressed the carbohydrate moiety at 24°C and 37°C and moreover, a newly synthesized protein epitope was only recognized at 37°C. In the light of our results these antigens (180 & 140 kDa) showed different response mechanisms against the heat shock involving glycosylation process [25] and protein synthesis [2,5].

To investigate the possible location of cell wall HSAs (223, 205, 180 and 140 kDa) whose expression was enhanced in UPV RSI extracts after heat shock, a double adsorption (Figure 6, lines DA) of saliva was performed. Generally, all the bands showed a decrease in the intensity but the 140 kDa antigen was still visible at 37°C in both extracts after the second adsorption. This demonstrates that heat shock antigens identified were localized to the cell wall surface of *C. albicans* or at least were accessible to the antibodies, but after heat shock an internal expression of the 140 kDa antigen, corresponding to a proform of the same antigen expressed at 24°C or to a novel protein synthesis, could be observed (Figure 4). This is not the first example describing a functional HSP in the *C. albicans* cell wall. Like mannoprotein antigens [14] or those related to HSP 70 [2], we prove in this study the cell wall location of a HSP 140 reacting with IgA, studies are underway to determine whether these proteins belong to the same family of HSPs. Moreover, the HSAs of HMW that were found were not recognized by salivary IgG antibodies. The lack of a reaction against IgG confirms the importance of IgA in secretions and in the defense at the mucosal level, also this protective effect of IgA has been found against other bacterial endotoxins as in *Clostridium* [10].

In recent studies [26-28] it has been proved that glycosidic residues of a 200 kDa heat shock glycoprotein (HSGP) from *C. albicans* yeast cell wall act as a haemolytic factor and a macrophage stimulating signal in that it enhances the tumor necrosis factor (TNF) activity of both human and murine macrophages. These studies are based on a 200 kDa region previously described by our group [14]; a partial characterization of this HMW defined band allows us to know which of these bands are really responsible for the macrophage stimulation.

Antigens of similar MW have been defined by other investigators from the mycelial phase of *C. albicans* [23,29-31]. Although HSP of 70 and 90 kDa have been characterized by different groups [2,32,33], and we have found antigens of these MWs, that have not detected an IgA response in salivary samples.

In addition to temperature-regulated antigens we identified a non temperature regulated 61 kDa antigen (Figure 2). The 61 kDa antigen lost their expression after infection in the UPV RS1. This antigenic shift may be the result of a conformational change in the protein structure during the infection. Antigenic variability has also been described in *Candida* [34] and in other microorganisms [35]. Protein modifications induced by heat shock are known, such as glycosylation, acetylation, and phosphorylation [25]. Bensaude *et al.* found a new phosphorylation process in a 140 antigen but we do not know if it was the same protein described in the paper. Studies with 2D PAGE and western blot are in progress to elucidate this question. Moreover, other changes due to the stress induced by infection could be observed (Figures 1 & 2) and the influence of glycosylation seems to be an important mechanism in the recognition by secretory antibodies (Figure 4).

Antigens of relative low molecular weight showed reactivity against IgG (Figure 7), although the protein concentration was almost four times lower in these slabs gel because of the higher expression of these proteins. No HSAs recognized by IgA were found in the region. However, the 61 kDa protein from RSI strain shows that infection induces changes in the protein other than heat shock and that could be important in the infective process.

The yeast phase is the major form in which *C. albicans* survives as a saprophytic microorganism in the host. An alteration in the host could produce the stress response in *Candida* synthesizing these HSPs that, as a result, would raise the fungi adhesiviness to host mucous membranes. The existence of normal titers of IgA in saliva of healthy individuals would opsonize these proteins. They could even be separated from the mucousa by saliva washes, avoiding the adhesion and the possibility of an infection. To proove this hypothesis is the objective of our next series of experiments.

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