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Identification of cell surface determinants in *Candida albicans* reveals Tsa1p, a protein differentially localized in the cell

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Abstract To identify cell surface proteins of *Candida albicans*, the predominant fungal pathogen in humans, we have established an approach using a membrane impermeable biotin derivative in combination with affinity purification. We were able to identify 29 different proteins under two distinct conditions. Among mannoproteins, heat shock proteins and glycolytic enzymes we found thiol-specific antioxidant-like protein 1 (Tsa1p) to be differentially localized depending on the conditions applied. Only in hyphally grown cells Tsa1p was localized to the cell surface whereas in blastospores no surface but mainly nuclear localization was found. This indicates that cell surface expression of at least some proteins is mediated by differential translocation.

Key words: *Candida albicans*; Cell surface protein; Surface biotinylation; Affinity chromatography; Thiol-specific antioxidant-like protein

1. Introduction

During the past years there has been an increase of fungal infections in humans predominantly caused by *Candida albicans* [1]. This facultative pathogen coexists in the majority of the population as a commensal. But particularly in immunocompromised patients, the sensitive relationship of the host and the fungus is out of balance. This may result in life-threatening, systemic infections. Major virulence traits of *C. albicans* are the ability to switch from growth of singular budding cells (blastospores) to a filamentous growth form (hyphae), called the dimorphic transition, as well as adhesion to host tissues mediated by cell wall components [2,3].

The composition of fungal cell walls is highly dynamic. The cell wall has been implicated in several physiological processes, including the maintenance of cellular morphology and osmotic protection of the cell [4,5]. In addition to these essential functions, the cell wall of fungal pathogens is crucial for virulence. Antigenic determinants which are involved in adhesion and therefore colonization and modulation of the immunological response by the host contribute to pathogenesis [1,6]. Moreover, the absence of the cell wall in mammalian cells makes it an attractive target for the development of novel antifungal substances [1,6]. In general, the cell wall is composed of complex polymers of glucose (β-1,3- and β-1,6-glucan), chitin and mannoproteins. These components interact to give rise to the overall architecture [7]. Proteins either are covalently linked to the glucan network or are non-covalently incorporated into the cell wall [8,9]. Covalently linked cell wall proteins can be released from walls by treatment with specific hydrolases. Non-covalently-linked or sodium dodecyl sulfate (SDS)-extractable cell surface proteins can be released by using detergents [5]. Only little is known about the structure of non-covalent attachments of proteins to the cell wall.

To investigate cell surface proteins in fungi several proteomic approaches have been established both in *Saccharomyces cerevisiae* and *C. albicans*. Purified cell walls were most widely used to subsequently isolate attached proteins. Glucanase digests, as well as the use of reducing agents or proteases, led to the identification of novel cell wall proteins [10–13]. Specific labeling of cell surface proteins using a membrane impermeable biotin derivative has been used successfully to identify several proteins with internal repeats (Pir) and new cell wall glucanases in *S. cerevisiae* [14,15]. In *C. albicans* similar approaches were used to analyze cell wall dynamics [16] and Pir proteins [17].

We have established an approach using sulfosuccinimidyl-6-(biotinamido)hexanoate (Sulfo-NHS-LC-Biotin) to specifically label cell surface proteins of intact cells. We extracted these proteins and purified them by affinity chromatography. In total we identified 29 different cell surface proteins from *C. albicans* under two distinct conditions. For thiol-specific antioxidant-like protein 1 (Tsa1p), one of the identified proteins, we could show differential localization depending on growth conditions. Tsa1p could be detected at the cell surface, in the cytoplasm and in the nucleus.

2. Materials and methods

2.1. Strains and media

C. albicans strain SC5314 (wild-type) was precultured at 30°C in 10 ml YPD (1% yeast extract, 2% bacto peptone, 2% glucose) overnight. Fresh cultures were inoculated with overnight culture in a dilution of

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Abbreviations: AhPC, alkyl hydroperoxide peroxidase C; BSA, bovine serum albumin; Gfp, green fluorescent protein; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; α-MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline; Pir, protein with internal repeats; POD, horseradish peroxidase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tsa1p, thiol-specific antioxidant-like protein 1; YPD, yeast peptone dextrose

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1:100 and grown for 24 h either in YPD medium at 30°C as blastospores or in Eagle's minimal essential medium (α-MEM) +2% glucose at 37°C to induce hyphae. For analytical experiments 10 ml of cultures and for preparative approaches 1800 ml of cultures were harvested. *C. albicans* uridine-auxotrophic strain CAI4 [18] was used for transformations. Strain TG 6 (*TSA1*/*TSA1*::*GFP-URA3*) was derived from CAI4 as described in 'plasmids and transformations of *C. albicans*'.

2.2. Biotinylation and affinity purification of cell surface proteins

After three washing steps in cold phosphate-buffered saline (PBS) pH 7.4 cells were incubated for 2 h at 4°C with 10 mg ml^{-1} Sulfo-NHS-LC-Biotin (Molecular Probes, Eugene, OR, USA) in binding buffer, 50 mM $NaHCO_3$ pH 8.5. Remaining reactive Sulfo-NHS-LC-Biotin was blocked by adding two volumes of 100 mM Tris–HCl pH 8.0 and further incubation for 1 h. Cells were harvested by centrifugation at 3500×*g* and washed three times in cold PBS pH 7.4. For analytical experiments labeled cells were disrupted by vortexing two times for 5 min at 8°C using two volumes glass beads (0.25–0.5 mm; Roth, Karlsruhe, Germany) and one volume PBS pH 7.4 containing 1% Nonidet P-40 (NP-40) and 0.1% SDS. For preparative approaches disruption was done by using three cycles of French Press at a pressure of 1.5×10^6 hPa (SLM Instruments, Champaign, IL, USA) using the disruption buffer described above. Extracts were centrifuged at 3500×*g* for 5 min at 4°C and supernatants were centrifuged at 16 000×*g* for 10 min. Biotinylated proteins were purified by affinity chromatography using neutravidin (Molecular Probes, Eugene, OR, USA) coupled to Sepharose. For this purpose 3–4 mg neutravidin per ml matrix were coupled to CNBr (cyanogen bromide)-activated Sepharose 4B (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's protocol. Neutravidin Sepharose was equilibrated in PBS containing 1% NP-40 and 0.1% SDS prior to use. One ml extract from biotinylated cells per 100 μl of immobilized neutravidin were incubated at 4°C overnight. After binding, Sepharose beads were washed four times in cold PBS containing 1% NP-40 and 0.1% SDS and eluted with sample buffer for 5 min at 55°C. For analytical purposes bound proteins were eluted using 1 mM d-Biotin in *N*,*N*-dimethyl formamide.

2.3. Gel electrophoresis and Western blotting

Affinity purified cell surface proteins were precipitated by acetone before separation by SDS–polyacrylamide gel electrophoresis (PAGE) as previously described [19]. Affinity purified proteins were separated by SDS–PAGE using 10% gels. Silver and Coomassie staining of protein gels was performed as previously described [20]. For Western blotting proteins were transferred onto PVDF membrane (Immobilin-P, Millipore, Bedford, MA, USA) using a semi-dry blotting apparatus. Subsequently, the membrane was blocked for 1 h in PBS pH 7.4 containing 5% milk powder and washed three times for 10 min in PBS containing 0.05% Tween 20 (PBST). Proteins were detected by either probing with streptavidin conjugated to horseradish peroxidase (POD, Pierce, Rockford, IL, USA) diluted to a final concentration of 0.5 μg ml^{-1} in PBST containing 1% bovine serum albumin (BSA) for 1 h, or using polyclonal antisera directed against green fluorescent protein (Gfp; Clonetech, Palo Alto, CA, USA) diluted to a final concentration of 1 μg ml^{-1}. Anti-Gfp antibodies were detected by goat anti-rabbit IgG conjugated to POD (Jackson, West Grove, PA, USA) Blots were developed using ECL (enhanced chemiluminescence, Amersham Pharmacia) and documented using a LAS-1000 CCD camera (Fujifilm, Elmsford, NY, USA).

2.4. Identification of proteins

For identification of proteins major Coomassie-stained bands were excised from the gel and digested using trypsin for 6 h at 37°C. Resulting peptide fragments were eluted three times with 0.1% trifluoric acid in 60% acetonitrile. The eluted fractions were pooled, lyophilized and resuspended in 20 μl formic acid and 80 μl distilled water. In case of Tef1p, Gap1p and Adh1p the peptides were separated on a reversed phase liquid chromatography (Smart System, Amersham Pharmacia) with 2×100 mm RP 18/110/2 μm (TSK Super ODS) applying a gradient of 3–60% acetonitrile containing 0.1% trifluoric acid. The mass of separated peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (GSG Future, Bruchsal, Germany) and amino acid sequences were determined by microsequencing. The other proteins were also digested using trypsin and identified by peptide mass fingerprinting.

2.5. Indirect immunofluorescence

Cells were harvested after cultivation, washed once and fixed in PBS pH 7.4 containing formaldehyde (3.7%) for 10 min at room temperature and centrifuged at 380×*g*. Cells were resuspended in PBS pH 7.4 with 1 mg ml^{-1} BSA (PBS/BSA) and incubated for 1 h. For visualization of biotinylation, monoclonal antibodies directed against biotin (BN-34, Sigma, Oberkochen, Germany) were applied in a final concentration of 43 μg ml^{-1} for 1 h, followed by three washing steps in PBS/BSA. Sheep anti-mouse antibody conjugated to Cy3 (Sigma) was diluted to a final concentration of 20 μg ml^{-1}. Cells were washed three times in PBS/BSA and immobilized on poly-lysine-coated glass slides. Excitation filter and barrier setups were according to [21]. Specimen were investigated by using a confocal laser scanning microscope (Leica TCS SP2).

2.6. Plasmids and transformation of C. albicans

Previously described methods were applied to generate a Tsa1p–Gfp fusion [21]. Briefly, the following primers were used to PCR-amplify the *GFP*-cassette from plasmid pMG 1506 [21]: Forward: TGGCACCCAGGTGATGAAACCATCAAGCCAAGCCCAGAA-GCATCCAAG-GAATACTTCAACAAAGTCAACAAAGGTGGT-GGTTCTAAAGGTGAAGAATTATT. Reversed: AAAATGCAA-TCTATTTGCTATGAAACCATAACGAAAAAAAAAAACGTA-CA-TATATATCTATAAATCCTGTTTATCTAGAAGGACCACCTTT-GATTG.

Ten PCR reactions of 100 μl were pooled, extracted once with chloroform, precipitated with ethanol and resuspended in 100 μl H_2O. Strain CAI4 [18] was used for transformation according to previous reports [22], resulting in strain TG 6. Insertion of *GFP* was confirmed by Southern blotting.

2.7. Binding experiments

We prepared cytosolic extracts from wild-type cells (SC 5314) and strain TG 6 expressing a Gfp-tagged Tsa1p. Equal amounts from hyphal wild-type cells (0.3 g wet weight) were incubated either with cytosolic extracts (~500 μg of total protein) from strain TG 6 prepared in 50 mM $NaHCO_3$ pH 8.5 (binding buffer) or with binding buffer for 3 h at 4°C. After three washing steps in cold PBS pH 7.4 cells were biotinylated, the labeled proteins were extracted, affinity purified and separated by SDS–PAGE. To compare protein patterns the cytosolic extract was also biotinylated and analyzed in the same way. A surface protein preparation was performed with strain TG 6 using equal amounts of hyphae (0.3 g wet weight). 1/50 of the cytosolic extract used for the incubation and 1/6 of the isolated cell surface proteins were applied on the gel. This gel was blotted and the membrane was probed with polyclonal antisera directed against Gfp as described above. As a loading control blots were probed using streptavidin–POD.

Blastospores (SC 5314) were also incubated with a cytosolic extract from wild-type cells before biotinylation. Cell surface proteins were isolated as described before.

3. Results

3.1. Labeling and purification of cell surface determinants in C. albicans

In order to identify cell surface proteins of *C. albicans*, non-covalently linked to the cell wall, we developed an approach to specifically label and subsequently purify these proteins by affinity chromatography. For this purpose we used an activated biotin derivative (Sulfo-NHS-LC-Biotin) that specifically reacts with basic amino acid residues of proteins and that is not able to penetrate membranes [15,16,23]. The labeling of surface determinants and membrane impermeability of Sulfo-NHS-LC-Biotin was confirmed by electron microscopy using a monoclonal antibody directed against biotin (data not shown). Biotinylated cells were harvested, disrupted and biotinylated proteins were purified in a single step by affinity

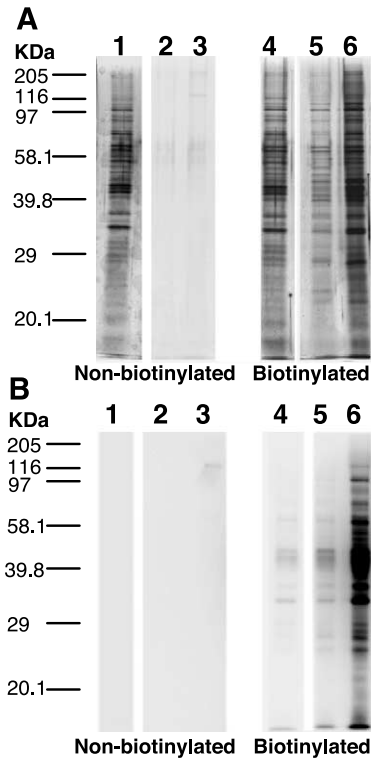


Fig. 1. Enrichment of biotinylated proteins. Wild-type cells (SC 5314) were labeled using Sulfo-NHS-LC-Biotin or incubated in buffer. Non-covalently linked proteins were extracted and applied to neutravidin Sepharose. Bound proteins were eluted by an excess of biotin or sample buffer and separated by SDS–PAGE, and were silver-stained (A) or subsequently blotted (B). The blot was probed using streptavidin–POD. Proteins were extracted and analyzed either from non-biotinylated cells (lanes 1–3) or from biotinylated cells (lanes 4–6). Crude extracts (lanes 1 and 4), elution with biotin (lanes 2 and 5), and elution with sample buffer (lanes 3 and 6).

chromatography using neutravidin Sepharose. Bound proteins were eluted using sample buffer revealing a particular protein pattern as determined by silver staining of SDS–PAGE (Fig. 1A, lane 6) as well as Western blotting (Fig. 1B, lane 6). From cell extracts of non-biotinylated cells only very few proteins could be isolated (Fig. 1A, lanes 2 and 3). At least one of these proteins with an apparent molecular weight of 120 kDa was biotinylated in non-biotinylated cells as shown by Western blotting (Fig. 1B, lane 3). This protein has been identified as pyruvate carboxylase (Pyc2p, Fig. 1A,B and Table 1, Y1 and H1, respectively), an enzyme with biotin bound as a cofactor.

Elution of affinity-purified proteins from biotinylated cells resulted in a well-resolved protein pattern (Fig. 1A, lanes 5 and 6), very similar to the pattern detected by Western blotting using streptavidin–POD (Fig. 1B, lanes 5 and 6). In contrast, no comparable pattern from non-biotinylated cells could be observed indicating that enrichment of surface proteins specifically depended on their biotinylation (Fig. 1B). This was further confirmed by the fact that qualitatively the same protein pattern was obtained by elution using an excess of free biotin (Fig. 1A,B, lane 5). However, less amount of protein was eluted by biotin competition compared to elution using sample buffer (containing SDS and reducing agent) which dissociated proteins more efficiently (Fig. 1A,B, lanes 5 and 6). No significant unspecific binding of proteins to the affinity matrix under the applied conditions was observed (Fig. 1A,B, lanes 2 and 3).

To identify cell surface proteins cells were grown in YPD at 30°C or α-MEM at 37°C, respectively. Cells were subsequently harvested and protein purification was scaled up as described in Section 2 to obtain sufficient amounts of cell surface proteins for their identification (Fig. 2A,B). All major bands of these preparations were excised from the gel. The bands indicated in Fig. 2 could be identified either by peptide mass fingerprinting or by microsequencing of internal peptides (Adh1p, Gap1p and Tef1p). In total, proteins from 39 different bands have been identified, 17 different proteins from blastospores grown in YPD at 30°C (Table 1, left) as well as 17 different proteins from hyphal cells grown in α-MEM at 37°C (Table 1, right), including Pyc2p found in both cultures. As Pyc2p has biotin bound as a cofactor we presume that purification of this protein was due to its in vivo biotinylation rather than being surface localized. The identified proteins can be assigned to different groups: Two cell wall mannoproteins, six glycolytic enzymes, five heat shock proteins, three phosphorylases, three elongation factors, and 10 proteins with varying functions (Table 1). Three proteins were found in both growth forms: Heat shock protein (Hsp70p), translational elongation factor 1 alpha (Tef1p) and glyceraldehyde dehydrogenase (Gap1p).

The two mannoproteins, pH-responsive antigen 1 (Pra1p, Table 1, H7) and pH-regulated protein 1 (Phr1p, Table 1, H3), were exclusively found in hyphae-inducing medium α-MEM at 37°C that has a pH of 8. This is in good agreement with reports showing that Phr1p is solely expressed in neutral to alkaline milieu [24] and that Pra1p is a very abundant cell wall protein in hyphal cultures [25]. Among those proteins identified from hyphae we found Tsa1p (Table 1, H17/18). Tsa1p is a member of the TSA/alkyl hydroperoxide peroxidase C (AhPC) family and yet not known to be surface localized.

3.2. *Tsa1p is differentially localized*

In order to confirm surface localization of Tsa1p in hyphae, we fused Gfp to its C-terminus. Using confocal laser scanning

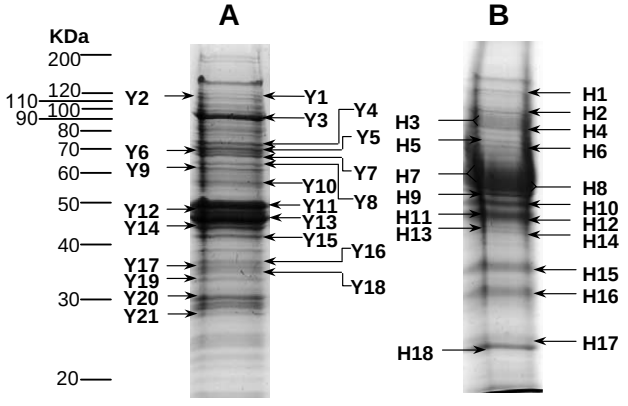


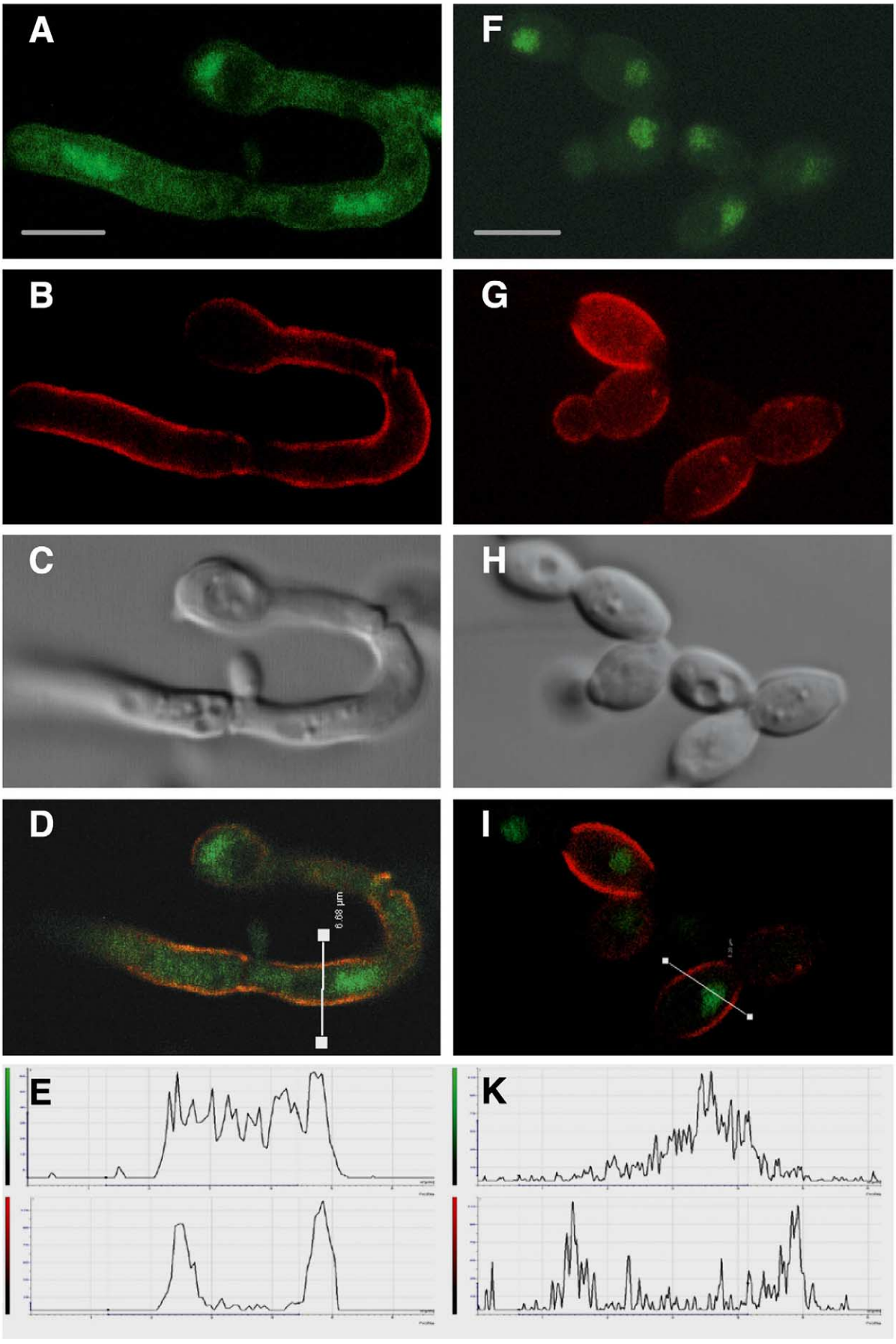
Fig. 2. Preparative purification of cell surface proteins from wild-type cells of *C. albicans* (SC 5314). Cell surface proteins isolated from blastospores (A) and hyphal cells (B). Proteins were purified as described, separated by 10% SDS–PAGE and subsequently stained by Coomassie. Each protein band marked with an arrow and a number was cut out from the gel, digested and identified either by peptide mass fingerprinting or microsequencing of internal peptides. See Table 1 for identities.

Table 1
Identified cell surface protein in *C. albicans*

Identified proteins YPD 30°C				Identified proteins α-MEM 37°C			
Band[a]	Swissprot/TREMBL, Acc. No., ORF[b]	MW[c] (kDa)	Function/homolgue[d]	Band[a]	Swissprot/TREMBL, Acc. No., ORF[b]	MW[c] (kDa)	Function/homolgue[d]
Y1	CA1463	109	Pyruvate carboxylase (Pyc2p)	H1	CA1463	109	Pyc2p
Y2	P25997	117	Translational elongation factor 3 (Eft3p) [26]	**H2**	**6.7947**	**103**	**Glycogen phosphorylase (Gph1p) homolog**
Y3	O13430	92	Translational elongation factor 2 (Eft2p) [26]	H3	P43076	60	pH-responsive protein 1 (Phr1p) [24]
Y4	**O94039**	**74**	**Transketolase (Tkt1p)**	H4	P46598	80	Heat shock protein Hsp90p [32]
Y5	P41797	70	Heat shock protein Hsp70p [34]	H5	U25718	70	Heat shock protein Ssa2p [34]
Y6	**CA4474**	**70**	**Heat shock protein Ssc1p**	H6	P41797	70	Heat shock protein Hsp70p [34]
Y7	6.5819	66	Heat shock protein Ssb1p [26] homolog	H7	P87020	34	pH regulated antigen (Pra1p) [25]
Y8	**CA4474**	**63**	**Heat shock protein Ssc1p**		6.4386	63	Pyruvate decarboxylase (Pdc11p) homolog [26]
Y9	P46614	56	Pyruvate kinase (Pyk1p) [33]	**H8**	**6.2580**		**Ubiquitin carboxyl-terminal hydrolase (Ubp1p) homolog**
Y10	**6.1349**	**53**	**UTP-glucose-1-phosphaturidyl transferase (Ugp1p) homolog**	H9	6.7003	49	**ATPase alpha chain (Atp1p) homolgue**
Y11	P16017	50	Translational elongation factor 1 alpha (Tef1p) [26]	H10	P16017	50	Tef1p [26]
Y12	P16017	50	Tef1p [26]	**H11**	**Q9P842**	**42**	**S-adenosylmethionine synthetase (Sam2p)**
Y13	P16017	50	Tef1p [26]	H12	P46273	42	Phosphoglycerate kinase (Pgk1p) [35]
Y14	P16017	50	Tef1p [26]	**H13**	**6.893**	**35**	**Echinocandin-binding protein (Ebp1p)**
Y15	O93827	40	GDP-mannose-pyrophosphorylase (Srb1p) [26]	H14	6.4310	38	Cell surface hydrophobicity protein (Csh1p) [37]
	P43067	40	Alcohol dehydrogenase (Adh1p) [33]	H15	Q92211	37	Gap1p [36]
Y16	**6.1494**	**37**	**Coprogen oxidase (Hem13p) homolgue**	H16	P16017	50	Tef1p [26]
Y17	P16017	50	Tef1p [26]	**H17**	**Q9Y7F0**	**22**	**Thiol-specific antioxidant protein (Tsa1p)**
Y18	Q92211	37	Glyceraldehyde-3-phosphate dehydrogenase (Gap1p) [36]	**H18**	**Q9Y7F0**	**22**	**Tsa1p**
Y19	AAB96910	30	14-3-3 protein (Bmh1p) [26]				
Y20	**6.4711**	**23**	**S10 ribosomal protein (Rps6p) homolog**				
Y21	**Q9P835**	**31**	**Succinate dehydrogenase (Sdh2p)**				

[a]Identifier of the corresponding bands in Fig. 2.
[b]Listed are either Swissprot/TREMBL numbers, GenBank accession numbers (Acc. No.) or the numbers of open reading frames (ORF) of annotation 6 from the Candida Genome Database (http://www-sequence.stanford.edu/group/candida).
[c]Molecular weight.
[d]Homolgues in other organisms. Proteins in bold letters were first discovered to be surface localized in this study. The other proteins have already been identified from cell wall isolations or have been detected at the cell surface by immunocytochemistry in *C. albicans* (references in brackets).

microscopy Tsa1p–Gfp could be detected in both yeast form and hyphal cells (Fig. 3A,F). However, only in hyphae Tsa1p could be localized to the cell surface. Cells expressing Tsa1p–Gfp were biotinylated and tagged cell surface proteins were labeled using monoclonal antibody directed against biotin (Fig. 3B,G). Superimposition of signals from Tsa1p–Gfp and Cy3-labeled cell surface proteins showed strong overlap in hyphae (Fig. 3D). This is also evident from the corresponding emission profile (Fig. 3E). In blastospores we could not detect Tsa1p–Gfp at the cell surface (Fig. 3F,I,K). However, the majority of the fusion protein was localized to the nucleus, as verified by DAPI staining (data not shown). Although expressed both in YPD at 30°C and in α-MEM at 37°C, Tsa1p was only present at the cell surface under these hyphae-inducing conditions, confirming our biochemical finding that Tsa1p could only be identified from hyphal preparations.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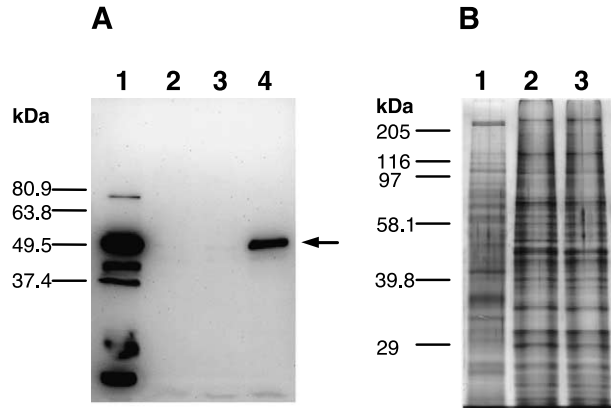

Fig. 4. Binding experiments on intact cells of *C. albicans*. A: Preparation of cell surface proteins from hyphal wild-type cells either incubated with binding buffer (lane 2) or incubated with cytosolic extract of strain TG 6, expressing a Tsa1p–Gfp fusion protein (lane 3); preparation of cell surface proteins from hyphal cells of strain TG 6 incubated with binding buffer (lane 4). After the incubations, cells were washed and biotinylated. Labeled proteins were extracted, affinity purified, separated by SDS–PAGE and transferred to PVDF membranes. The blot was probed using polyclonal antibodies directed against Gfp. The cytosolic extract from strain TG 6 used for the incubation was also biotinylated, affinity purified, and analyzed by Western blotting (lane 1; 10 μg of total protein applied). Bands with lower molecular weight in lane 1 represent protein degradation of Tsa1p–Gfp fusion protein and cross-reactivity of the antibody to cytosolic proteins. The band corresponding to the full-length Tsa1p–Gfp fusion protein is indicated by an arrow. B: Wild-type blastospores were either incubated for 3 h with cytosolic extracts (lane 2) or incubated in binding buffer (lane 3). After biotinylation, extraction and affinity purification, biotinylated proteins were separated by SDS–PAGE and silver stained. Aliquots of the cytosolic extract were also biotinylated, affinity purified and analyzed by SDS–PAGE (lane 1).

3.3. *Tsa1p–Gfp does not bind unspecifically to cell surfaces of* C. albicans

To avoid misinterpretation we have determined whether Tsa1p sticks unspecifically to the cell surface as it might be artificially released from the cytoplasm for example by cell lysis. For this purpose, we compared a preparation of surface proteins from wild-type hyphae incubated for 3 h with a cytosolic extract of strain TG 6 expressing a Tsa1p–Gfp fusion protein to a preparation of surface proteins from hyphal cells of strain TG 6.

Accordingly, wild-type cells (SC 5314) were grown in α-MEM medium at 37°C to induce hyphae. The hyphal cells were harvested and incubated for 3 h with cytosolic extracts from strain TG 6 expressing Tsa1p–Gfp. After this incubation, cells were washed and biotinylated. Cell surface proteins were purified, separated on SDS–PAGE and blotted to detect Tsa1p–Gfp. No signal could be observed (Fig. 4A, lane 3) indicating that Tsa1p–Gfp did not bind to the cell surface during the 3-h incubation period. In contrast, a preparation of surface proteins using strain TG 6 expressing Tsa1p–Gfp that was treated similar to the wild-type cells but without addition of cytosolic extract displayed Tsa1p–Gfp at the cell surface (Fig. 4A, lane 4). In order to determine the amount of Tsa1p–Gfp present in the cytosolic extract used for incubation, an aliquot of this extract was also analyzed by Western blotting (Fig. 4A, lane 1). To make sure that similar amounts of protein were applied to the blot, a Western blot using streptavidin–POD was performed to detect all biotinylated proteins as a whole (data not shown). Therefore, these results provide strong evidence that Tsa1p does not bind unspecifically to the cell surface of *C. albicans*.

To demonstrate more generally that cytosolic proteins do not bind unspecifically to the cell surface of *C. albicans*, we incubated wild-type cells grown as blastospores for 3 h with a cytosolic extract, prior to biotinylation. In parallel, another culture of wild-type blastospores was grown identically but incubated with binding buffer instead of cytosolic extract. After washing, both cultures were biotinylated, cell surface proteins were isolated and subsequently analyzed by SDS–PAGE (Fig. 4B). To determine the cytosolic protein pattern resulting from lysed cells, we also analyzed the cytosolic extract used to incubate the cells by SDS–PAGE and compared this pattern (Fig. 4B, lane 1) to the patterns of surface protein preparations described above (Fig. 4B, lanes 2 and 3). The protein patterns in lanes 2 and 3 showed no significant differences, neither qualitatively nor quantitatively. The pattern in lane 1 is clearly different indicating that the cell pattern of surface proteins (lanes 2 and 3) did not reflect the pattern of cytosolic protein extracts (lane 1). Hence, we conclude that surface localization of the proteins found by our approach was not due to unspecific binding of cytosolic proteins, for example from lysed cells.

4. Discussion

The cell surface of fungal pathogens is critical for adhesion to host tissues, representing an essential step during infection. Host–pathogen interactions are mainly mediated by cell surface proteins that are either covalently linked to the cell wall or are non-covalently incorporated [8]. Approaches to identify detergent-extractable cell surface proteins so far were based on isolation of cell walls and subsequent extraction of these proteins (e.g. [26]). The inherent vice of those approaches was that cytoplasmic contaminants could not be discriminated from cell surface determinants [5]. Due to this reason, we have specifically labeled cell surfaces of *C. albicans* using a membrane impermeable biotin derivative, Sulfo-NHS-LC-Biotin. Subsequently, non-covalently linked cell surface proteins were extracted from cell walls using detergent. We have first added a single step purification to separate the biotinylated proteins from non-labeled intracellular proteins by affinity chromatography using immobilized neutravidin. As biotinylated proteins were extracted simply by using detergents, covalently linked cell wall proteins were not purified by our

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Fig. 3. Subcellular localization of Tsa1p in blastospores and hyphal cells of *C. albicans* using confocal laser scanning microscopy. Gfp was fused to the C-terminus of Tsa1p for expression from its endogenous promoter. A–D: Hyphal cells were grown for 24 h in α-MEM at 37°C. F–I: Yeast cells were grown for 24 h in YPD at 30°C. A,F: Gfp signal. B,G: Surface biotinylation was visualized by monoclonal antibody directed against biotin and sheep anti-mouse antibody conjugated to Cy3. C,H: Transmission micrographs. D,I: Superimposition of Gfp and Cy3 signal. Corresponding emission profiles for hyphae (E) and blastospores (K). Upper graphs: Gfp signal; lower graphs: Cy3 signal; the *x*-axis represents white bars indicated in the corresponding superimpositions. Grey bars in A and F: 5 μm.

approach. We could identify by microsequencing of internal peptides and peptide mass fingerprinting 29 different proteins, 17 proteins from blastospores (YPD 30°C) and 17 proteins from hyphae (α-MEM 37°C), including Pyc2p, an enzyme with biotin as a cofactor. Pyc2p was isolated most likely due to this prosthetic group.

In contrast to previous reports that describe binding of proteins to immobilized avidins not mediated by biotin [27], we could not observe binding of non-biotinylated cell wall proteins of *C. albicans* to neutravidin under our conditions (Fig. 1A,B). Therefore, we concluded that unspecific and biotin-independent binding of cell surface proteins was significantly diminished.

4.1. Identified proteins

In our screen we found mannoproteins, heat shock proteins, glycolytic enzymes, pyrophosphorylases, elongation factors and other proteins with miscellaneous functions. Most of the proteins are known to be cytosolic missing secretion signal sequences. As glycolytic enzymes and heat shock proteins have also been localized to the cell surface of *C. albicans* by other groups alternative mechanisms for their subcellular distribution have been proposed [8]. In fact, protein export through non-classical secretory pathways has been demonstrated in *S. cerevisiae* and mammalian cells. For example, the a-factor of *S. cerevisiae* is transported to the cell surface by the ABC-transporter Ste6p [28,29]. Yeast cells export mammalian galectin, even if classical secretion is blocked [30].

Three lines of evidence strongly supported our findings that the isolated proteins represent bona fide cell surface determinants of *C. albicans*: (i) We have used an impermeable biotin derivative that exclusively labels cell surface proteins and purification of these proteins via neutravidin Sepharose was specific. (ii) In binding experiments we showed that cytosolic proteins in general and Tsa1p in particular did not adsorb unspecifically to the cell surface of living cells prior to biotinylation indicating that Tsa1p and the other proteins identified in this study are localized to the cell surface in vivo and are not derived from lysed cells, for example. However, in contrast to our approach binding of cytosolic proteins to isolated cell walls has been reported [31]. This indicates that intact cells which are covered with a coat of glycosylated proteins have a different affinity to proteins than isolated cell wall structures. (iii) We have isolated Tsa1p from cell surface preparations of *C. albicans* grown in α-MEM at 37°C but not in YPD at 30°C although this protein is abundant under both growth conditions. This is in good agreement with our finding that using confocal microscopy Tsa1p–Gfp fusion protein was exclusively localized at the cell surface when grown in α-MEM at 37°C. This indicates that the biochemical approach is specific for cell surface proteins.

Although we have isolated many proteins from either one of the two growing conditions we cannot exclude the possibility that proteins found solely under one condition are expressed also under the other. This may be the case e.g. for Hsp90p, which we could only identify in hyphae. However, Hsp90p has been detected on cell surfaces of hyphae and blastospores using a specific monoclonal antibody [32]. This points to the fact that the identification of proteins from the gels is not complete. However, the differences in protein patterns that we have observed indicate that there are differences in the protein expression under both conditions. Therefore, these differences might reflect an adaption to varying temperature, media or growth forms.

Many proteins found by our screen have already been isolated from cell walls of *C. albicans* or were detected at the cell surface using immunocytochemistry [8,26,33–37]. Enzymatic activity of phosphoglycerate kinase could be detected in suspensions of whole cells but not in the culture supernatant indicating a surface-dependent function [35]. Elongation factors have additionally been found to be cell surface proteins of *Mycobacterium leprae* [38] and of tobacco where vitronectin-binding properties of this protein were proposed [39]. Among those proteins that have yet not been described as surface determinants we found Tsa1p. Two separated bands of Tsa1p could be observed in SDS gels (Fig. 2B, H17/18) which might reflect partial degradation.

4.2. Possible functions of Tsa1p

By immunocytochemistry Tsa1p was shown to be differentially localized to the cell surface in *C. albicans* depending on the growth conditions. To test whether Tsa1p–Gfp was secreted we compared culture supernatants of strain TG 6 to the respective intracellular and cell surface-associated Tsa1p–Gfp from disrupted cells in a Western blot experiment. Less than 0.4% of total Tsa1p–Gfp was found in the culture supernatant (data not shown). Hence, we concluded that small amounts of Tsa1p might be released to the culture supernatant by lysis of cells rather than by secretion of the protein.

Tsa1p is a member of the TSA/AhPC family. Homology searches revealed significant homologies of Tsa1p to eukaryotic proteins involved in regulation of cellular proliferation and differentiation processes. For example, Tsa1p shares 55% identity with the product of human proliferation-associated gene (*pag*) [40]. Also a cysteine-rich 29-kDa surface antigen of *Entamoeba histolytica* which exhibits thiol-dependent peroxidase activity is 43% identical to Tsa1p [41,42]. In *S. cerevisiae* these enzymes function as thiol-peroxidases which eliminate reactive oxygen species, like H_2O_2 and alkyl hydroperoxides [43]. Interestingly, in *S. cerevisiae* ScTsa1p has been found to be essential for the transcriptional induction of components of the thioredoxin system in response to H_2O_2 [44]. Due to the differential localization of Tsa1p and significant homologies to proteins involved in oxidative stress-signaling pathways, we assume a similar function of Tsa1p in *C. albicans*. However, it remains to be established what the signals are that regulate the differential translocation and therefore the function of Tsa1p.

In summary, using this approach we could analyze the composition of non-covalently linked cell surface proteins of *C. albicans* under two distinct conditions in a comprehensive context. The findings indicate that cytosolic proteins also may have functions in the cell wall. How these proteins are subcellularly distributed and which functions they fulfil is not clear. This work might serve as a promising starting point to investigate those proteins in more detail.

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