Differential Protein Synthesis in *Candida albicans* during Blastospore Formation at 24.5 °C and during Germ Tube Formation at 37 °C

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To identify proteins synthesized during blastospore to germ tube transformation in *Candida albicans*, membrane and cytoplasmic protein fractions labelled with ^{14}C were analysed by SDS-PAGE and autoradiography. Four cytoplasmic proteins were detected in pulse-labelled lysates prepared from cells forming blastospores and germ tubes at 37 °C, but not in pulse-labelled lysates prepared from cells forming blastospores at 24.5 °C. Three proteins detectable in 24.5 °C lysates were diminished in 37 °C lysates.

INTRODUCTION

Candida albicans is a dimorphic fungus, normally a commensal organism in man, but capable of causing serious disease in the immunocompromised host. The yeast predominates *in vitro* when grown at 24.5 °C and the mycelial precursor germ tubes are inducible with various stimuli at 37 °C (Lee *et al.*, 1975; Shepherd *et al.*, 1980; Soll *et al.*, 1981). This organism normally exists as a yeast in the gastrointestinal tract of man, whereas the mycelial form is usually associated with disease. This has prompted several investigations into the gene products responsible for morphogenesis (Manning & Mitchell, 1980; Brown & Chaffin, 1981; Brummell & Soll, 1982). All of these studies have used stationary phase cells resuspended in medium containing various forms of ^{35}S or ^3H with various isotopic labelling protocols. Resulting cell lysates prepared in several ways were analysed by one- or two-dimensional PAGE.

All of the above studies excluded an analysis of a low-speed pellet fraction of these lysates. Here we present a study which incorporates several significant variations in technique and which shows protein differences previously undetected between blastospore formation at 24.5 °C and germ tube formation at 37 °C. We will describe increased production of four new proteins at 37 °C. These results differ from the results of others who described blastospore-specific proteins but no mycelial-specific proteins (Brown & Chaffin, 1981). The discussion focuses on the results obtained using different labelling protocols.

METHODS

Organism and growth conditions. *Candida albicans* strain B311-10 was maintained at –70 °C on Sabouraud's dextrose agar. From the freezer, the organism was inoculated into the synthetic medium for *Candida* (SMC) of Lee *et al.* (1975), and maintained in early-exponential phase ($A_{675} < 0.5$) by repeated transfers into fresh SMC medium for 48 to 72 h at 24.5 °C, with gyratory shaking at 240 r.p.m. These growth conditions yielded a culture consisting solely of budding yeasts with minimal clumping.

Pulse labelling procedure. A 300 ml culture was grown for 18 h to a final density of $A_{675} = 0.5$. For the pulse labelling at 24.5 °C, 100 ml culture was removed, rapidly filtered, and washed twice with SMC prepared without leucine. The filter was resuspended in 100 ml SMC without leucine but containing 250 μCi of uniformly labelled L-[U- ^{14}C]leucine (340 mCi mmol $^{-1}$; 12.6 GBq mmol $^{-1}$). The cells were labelled for 15 min, put on ice with the addition of 130 mg dry L-leucine, and then rapidly filtered. The filter was washed twice with ice-cold 0.1 M-Tris/HCl buffer, pH 8.0, and twice with 0.1 M-Tris/HCl buffer, pH 8.0, containing 50 mM-DTT. The filter was

subsequently resuspended in 5 ml 0.1 M-sodium phosphate buffer, pH 6.8, containing 1 mM-phenylmethylsulphonyl fluoride (PMSF), 50 mM-DTT and 20 mg Zymolyase 5000. This mixture was incubated for 20 min at 30 °C with shaking and then placed on ice. The remaining 200 ml culture was added to 200 ml SMC that had been prewarmed to 37 °C, and this shaking culture was closely monitored for germ tube production. At the desired stage of germ tube formation 200 ml of this culture was removed, filtered, resuspended in medium and pulse-labelled as described above.

Protoplast lysates. The 5 ml suspensions of L-[¹⁴C]leucine labelled protoplasts were lysed by the addition of 45 ml ice-cold water containing 1 mM-PMSF. These were frozen at -70 °C to facilitate breakage. Upon thawing, all lysates were resupplemented to 1 mM-PMSF and all subsequent manipulations were performed at 2 to 4 °C. Lysates were centrifuged at 1500 g for 5 min, which reduced the number of unlysed cells to less than 0.05%. Each cleared lysate was centrifuged at 54000 g for 30 min, and the pellet was referred to as fraction 1. The supernatant was centrifuged at 105000 g for 120 min, and the pellet was referred to as fraction 2. The resulting supernatant was referred to as fraction 3. Pellets were resuspended in 10 ml water containing 1 mM-PMSF and all fractions were dialysed three times against 2 litres of 1 mM-ammonium bicarbonate buffer, pH 7.5, containing 1 mM-PMSF. Dialysates were shell frozen, lyophilized, and resuspended in water to an activity of 3000 cold TCA-insoluble d.p.m. µl⁻¹. Cold TCA-insoluble d.p.m. were measured by the addition of 25 µl lysate to 5 ml ice-cold 10% TCA. After 30 min incubation on ice, precipitates were filtered on GF/A glass-fibre filters (Whatman) and washed three times with 5 ml portions of ice-cold 10% TCA and twice with 5 ml portions of ice-cold 95% ethanol. The dried filter was incubated overnight with 1 ml NCS (Amersham) and subsequently counted in 4.5 ml of OCS toluene-based scintillation mixture (Amersham). Total soluble d.p.m. were measured by addition of 25 µl lysate to 5 ml ACS scintillation mixture (Amersham). All samples were counted in an Intertechnique 3000SL liquid scintillation counter. Lysate fractions were divided into portions and stored at -70 °C.

SDS-PAGE. Vertical slab gel (160 × 140 × 1.5 mm) analyses were performed using a discontinuous gel and buffer system. A sample containing 40000 d.p.m. was added to an equal volume of 0.125 M-Tris/HCl sample buffer containing 25% (v/v) glycerol, 2.5% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, and 0.0025% (w/v) bromophenol blue and was immediately denatured at 100 °C for 2 min. The running gel was 9% (w/v) acrylamide, 0.27% (w/v) *NN'*-methylenebisacrylamide 0.1% (w/v) SDS in 0.375 M-Tris/HCl buffer, pH 8.8. The stacking gel was 4.5% acrylamide, 0.12% *NN'*-methylenebisacrylamide, 0.1% SDS in 0.125 M-Tris/HCL buffer, pH 6.8. Gels were polymerized by addition of 0.033% (w/v) ammonium persulphate and 0.25% (w/v) *N,N,N,N*-tetramethylethylenediamine. The gels were electrophoresed in 0.025 M-Tris/HCl, 0.19 M-glycine, pH 8.3, containing 0.17% SDS. Gels were fixed and stained in 0.5% (w/v) Coomassie Brilliant Blue R-250 in 10% methanol and 10% glacial acetic acid for visualization of the non-radioactive molecular weight markers (Pharmacia). The distance of migration from the origin was plotted against the logarithm of the molecular weight for each standard protein and unknown values were interpolated from this curve. Destaining of gels was in 10% methanol, 10% glacial acetic acid. Gels were prepared for autofluororadiography by soaking in Enhance autoradiography enhancer (New England Nuclear) for 1 h and cold water for 1 h before being dried on a slab gel drier (Hoefer Scientific Instruments, San Francisco). Dried gels were exposed to Kodak X-O Mat R film at -70 °C for 6 to 15 d in a DuPont Cronex cassette. Developed autoradiographs were contact printed on Kodak FP6 paper.

Reagents. Zymolyase 5000 was obtained from Kirin Brewery, Japan. L-[¹⁴C]Leucine was obtained from New England Nuclear. Acrylamide and *N,N,N,N*-tetramethylethylenediamine were from BDH. All other electrophoresis reagents were obtained from Bio-Rad. Phenylmethylsulphonyl fluoride (PMSF) was obtained from Calbiochem-Behring. Amino acids for synthetic media were obtained from ICN Nutritional Biochemicals (Cleveland, Ohio). Distilled, deionized, reverse osmotic water used for all media and solutions was prepared by a Milli-Q system (Millipore).

RESULTS

Germ tube induction

When *C. albicans* B311-10 is grown in SMC at 24.5 °C, there are no hyphae, pseudohyphae or germ tubes present. When this culture of budding yeasts in exponential phase growth ($A_{675} = 0.5$) is diluted into an equal volume of SMC prewarmed to 37 °C, germ tubes are produced synchronously and reproducibly. Few or no germ tubes can be demonstrated before 45 min after the shift to 37 °C (Fig. 1). Then, in as little as 20 min, 50% of yeast cells develop the distinctive germ tube morphology. One hundred percent of cells are transformed to germ tube-bearing cells by 105 to 120 min after the temperature shift.

Distribution of label after fractionation

The total d.p.m. incorporated into washed cells were assayed immediately after centrifugal fractionation of the whole cell lysates. The distribution of label was constant in various fractions

Fig. 1. Time course of germ tube development after shift to 37 °C. The bars represent standard deviation of three separate experiments.

Table 1. *Distribution of total label after fractionation*

The cells were labelled with L-[¹⁴C]leucine for 15 min at 24.5 °C or 37 °C. Protoplasts were created using a procedure described in Methods. Osmotically lysed protoplasts were fractionated centrifugally and the fractions were assayed for incorporated radioactivity. The numbers in parentheses represent the percentage of the total radioactivity.

Fraction	10 ⁻⁷ × Radioactivity in lysate (d.p.m.)		
	24.5 °C blastospore	37 °C blastospore	37 °C mycelium
Low-speed	3.9 (31.2)	4.6 (31.1)	4.4 (27.0)
High-speed pellet	2.6 (20.8)	3.8 (25.6)	3.9 (23.9)
High-speed supernatant	6.0 (48.0)	6.4 (43.2)	8.0 (49.0)

from lysates labelled at different times (Table 1). As expected, the total uptake of isotope during a 15 min pulse at 37 °C was greater than the uptake at 24.5 °C.

Protein differences between 24.5 °C and 37 °C

An autoradiograph of the protein patterns generated by SDS-PAGE of the fractionated, labelled lysates prepared from cells grown at 24.5 °C and 37 °C is shown in Fig. 2. The first three lanes represent the low-speed pellet fractions from each of the three lysates; blastospores at 24.5 °C (A), blastospores after 25 min at 37 °C (B), and germ tubes after 65 min at 37 °C (C). The next three lanes (D-F) represent the high-speed pellets from these same lysates, and the last three lanes (G-I) represent the high-speed supernatants from these lysates.

As can be seen in the first fraction, there were no detectable protein differences between membranes from cells grown at 24.5 °C and those from cells grown at 37 °C. In the second fraction, only one protein difference was visible. A 31 000 molecular weight protein was a minor constituent at 24.5 °C, but became a well-resolved protein both in 37 °C blastospores and in cells which developed germ tubes at 37 °C.

Most of the important protein differences between the two temperatures were seen in the high-speed supernatant fractions. Three proteins showed increased production at 37 °C and three showed diminished production at 37 °C. Two proteins showed the same dramatic increase in production at 37 °C; a 40 000 and a 75 000 molecular weight protein were either non-existent or very minor constituents at 24.5 °C, but became well-resolved, significant proteins in both

Fig. 2. Autoradiography of SDS-PAGE analysis of pulse-labelled lysates of *C. albicans* at 24.5 °C and during germ tube formation at 37 °C. A-C, low-speed pellets of lysates from: 24.5 °C blastospores (A), 37 °C blastospores (B), 37 °C germ tubes (C). D-F, high-speed pellets of lysates from: 24.5 °C blastospores (D), 37 °C blastospores (E), 37 °C germ tubes (F). G-I, high-speed supernatants of lysates from: 24.5 °C blastospores (G), 37 °C blastospores (H), 37 °C germ tubes (I). The molecular weights designated on the right of the figure correspond to protein bands which are associated with the 37 °C-induced blastospore to germ tube transformation. The 31 000 protein seen in the 24.5 °C high-speed pellet, lane D, increases at 37 °C in lanes E and F. The 40 000 and 75 000 molecular weight proteins are well-resolved in the 37 °C supernatants, lanes H and I, but are not visible in the 24.5 °C supernatant, lane G. The 70 000 molecular weight protein visible in lane G increases significantly in the 37 °C supernatant, lane H, and further increases in lane I. The 56 000 and 78 000 molecular weight proteins seen in the 24.5 °C supernatants, lane G, decrease in the 37 °C supernatants, lanes H and I. The 42 000 molecular weight protein visible in lane G is not visible in lanes H and I.

37 °C blastospores and germ tubes. A 70 000 molecular weight protein was present at a barely detectable level in the 24.5 °C lysate, increased in 37 °C blastospores and increased further in 37 °C germ tubes. Of the three proteins which diminished in 37 °C lysates, two were major proteins (56 000 and 78 000 molecular weight) in the 24.5 °C lysate, which decreased detectably in both 37 °C blastospores and 37 °C germ tubes. A 42 000 molecular weight protein was a minor constituent of the 24.5 °C lysate which became undetectable in both 37 °C lysates.

In some experiments, lysates were prepared without the addition of the protease inhibitor, PMSF. The protein bands which were visualized after electrophoresis of these lysates were diffuse compared to the electrophoretic pattern resolved from lysates prepared in the presence of PMSF (data not shown here). In addition to the reduced clarity obtained from the unprotected lysates, fewer proteins were detected overall, the majority of this activity resided in the 20 000 to 40 000 molecular weight range, and a large amount of radioactivity lay unseparated at the bottom of the gel. Very few proteins were visualized in the 60 000 to 90 000 molecular weight range in these lysates compared to the many proteins which appeared in this region on gels made from lysates prepared with PMSF. Despite the reduced clarity of the unprotected lysates, protein differences were noted between 24.5 °C and 37 °C lysates. These protein differences were detected in the 35 000 to 50 000 molecular weight range. As discussed above, the inclusion of protease inhibitor yielded lysates which when electrophoresed demonstrated protein differences between 24.5 °C and 37 °C in the 30 000 to 80 000 molecular weight range.

DISCUSSION

In this study we have examined pulse-labelled lysates of *C. albicans* in search of phenotype-specific proteins. We have used a continuously growing exponential phase culture, pulsed a sample at 24.5 °C, then diluted the remainder into fresh medium at 37 °C, and pulsed a sample before and during germ tube formation. This methodology provided a highly reproducible and condensed time scale of germ tube development elicited by a differential temperature stimulus. Two of the recent studies on proteins synthesized during *Candida* morphogenesis have also used a temperature stimulus to induce the mycelial phase of growth (Brown & Chaffin, 1981; Manning & Mitchell, 1980). Like the data derived from these investigations, the present data cannot be used to distinguish temperature-dependent proteins from morphology-specific proteins. We will investigate further this relationship using a non-mycelium producing variant already described (Buckley *et al.*, 1982).

Manning & Mitchell (1980) used two-dimensional electrophoresis to analyse equilibrium-labelled cytoplasmic proteins from a mycelium producing and a non-mycelium producing strain of *C. albicans*. Extracts prepared from both strains grown at 24 °C and 37 °C were also cross-absorbed with antisera raised against each extract, and two-dimensional analyses of the remaining proteins were used to elucidate temperature-related and strain-related protein differences. These workers described ten mycelium-specific proteins of the following approximate molecular weights: 15 500, 26 500, 29 000, 29 500, 29 500 (different isoelectric points), 31 000, 31 500, 34 000, 40 000 and 49 000. Thirty-three yeast phase specific proteins were cited, but data sufficient to calculate molecular weights was not shown.

Two of the proteins which we have described as increasing in the lysates prepared from cells grown at 37 °C, have molecular weights which are in agreement with molecular weights of mycelium-specific proteins described by Manning & Mitchell; namely, a 31 000 protein seen to increase in our high-speed pellet and a 40 000 protein seen to increase in our high-speed supernatant. Although molecular weight data for the 33 yeast-specific proteins were not given, overall the molecular weights of protein differences cited by Manning & Mitchell were lower than the molecular weights of relevant proteins described in this study. We have described two additional mycelium-associated proteins (70 000 and 75 000 molecular weight) which were not seen by these workers. A major difference between our protocol and that of Manning & Mitchell is our use of a pulse labelling procedure.

Experiments conducted by Brown & Chaffin (1981) did use a pulse labelling format in a temporal analysis using two-dimensional gel electrophoresis. These workers also resolved over

200 protein spots in each gel. Four lysates were labelled at intervals during growth at 25 °C and 37 °C. Comparisons between proteins labelled at the two temperatures demonstrated seven proteins which were associated with the yeast phase. These proteins had the following approximate molecular weights: 40 000, 58 000, 59 000, 75 000, 82 000, 85 000 and 96 000. These proteins were in the same molecular weight range as the pertinent protein differences shown in our study. All three of the yeast-associated proteins we describe have a relationship with one of these proteins. The 40 000 molecular weight protein found only in the yeast by Brown & Chaffin may be the 42 000 molecular weight protein we describe as a minor protein at 24.5 °C and which became significantly reduced at 37 °C. The 56 000 molecular weight protein we have seen to be a major yeast component with a reduced presence at 37 °C may be either of two proteins described by these workers; a 58 000 molecular weight protein visualized only in yeast and early mycelial extracts or a 59 000 molecular weight protein only visualized in the yeast (Brown & Chaffin, 1981). The 78 000 protein, which we show as a major yeast protein that diminishes at 37 °C, may be either their 75 000 or 82 000 molecular weight protein, both of which they described as major yeast proteins and minor mycelial proteins. This investigation did not include an analysis of the lysate pellet generated by centrifugation at 15 000 *g* in the case of the spheroplast lysate and at 10 000 *g* in the case of the homogenized lysate. A major discrepancy arises due to our finding of four mycelium-associated proteins, none of which was found by these other workers.

In a recent study of temporal regulation of protein synthesis in *C. albicans*, Brummell & Soll (1982) used zinc-depleted, stationary phase cells resuspended in medium of different pH values at 37 °C. They found that each pH supported one of the two growth forms of this organism. Their temporal, one-dimensional, SDS-PAGE analysis of nine pulse-labelled lysates, prepared at 30 min intervals, described five proteins which were differentially synthesized late in both filamenting and budding cells. Two of the differentially synthesized proteins which we have described have molecular weights which correspond to the molecular weights of these proteins. Brummell & Soll (1982) also analysed selected lysates by two-dimensional gel electrophoresis and over 250 spots were distinguishable for both filamenting and budding populations. This yielded only one additional spot for each morphology, an approximately 48 500 molecular weight protein in the budding cells and an approximately 47 500 molecular weight protein in the mycelial cells. This result correlates with one of the 10 mycelial proteins seen by Manning & Mitchell (1980), but does not correlate with any of the budding proteins seen by Brown & Chaffin (1981). The data from their study is uncomplicated by temperature-related differences, but any variations due to the pH difference are likewise unresolved. These workers have shown an initial lag during which there is no protein synthesis. Hypothetically, this has been explained as being due to a requirement for zinc. The low-speed pellet generated by centrifugation at 8 000 *g* was not included in the SDS-PAGE analysis.

The investigation reported here differs from the others discussed above in several significant respects. Our system is the only one which used a continuously growing, exponential phase culture. Consequently, we did not experience the lag expected to occur in any procedure which used stationary phase cells to induce germ tube formation.

Our use of uniformly labelled L-[¹⁴C]leucine has generated good resolution of pulsed proteins in short exposure times by enhanced autoradiography of SDS-PAGE. The isotope eliminates any selective disadvantage in visualizing proteins low in sulphur content. The two-dimensional analysis used by others has the benefit of potentially greater resolution, but we have expanded the sensitivity of our one-dimensional analysis by fractionating all lysates. We have also included the low-speed pellet in our analysis. This fraction has not shown any significant protein differences between cells grown at 24.5 °C and those grown at 37 °C.

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