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Alteration of Ergosterol Content and Chitin Synthase Activity in *Candida albicans*

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Abstract. Chitin synthase activity in total membrane fractions from two polyene-resistant, ergosterol-deficient mutants of *Candida albicans* was significantly higher in comparison to the parental polyene-sensitive strain. The zymogen component from membrane preparations of stationary-phase cells of ergosterol mutants was more susceptible to trypsin digestion than those from the parental polyene-sensitive strain. Mechanisms that might explain the effect of changes in membrane composition in the mutant strains on chitin synthase activity are discussed.

Intensive studies on the mode of action of polyene antibiotics have shown that all fungi having sterols in their cell membranes are sensitive to polyene antibiotics, and close correlation was found between the amount of polyene bound and the ergosterol content of the yeast cells [9]. Consequently, the isolation of polyene-resistant mutants with altered membrane sterol content has become a useful tool for studying the importance of sterols in relation to the structure and function of membranes [13,17].

Alterations in the composition of the cell membrane may cause changes in the plasma membrane processes of *Candida albicans* [20], including the activities of membrane-bound enzymes. Chitin synthetase (EC 2.4.1.16) in *C. albicans* has been shown to be mainly associated with the plasma membrane [1], as had been earlier reported for *Saccharomyces cerevisiae* [5], and in both species it is involved in the formation of the primary bud scar.

Thus the purpose of the study reported in this paper was to compare the activity of chitin synthetase in an ergosterol-producing, polyene-sensitive strain of *C. albicans* and its polyene-resistant mutants, which do not contain ergosterol, in an attempt to assess changes in membrane activity with respect to chitin formation.

Materials and Methods

Microorganisms. Two polyene-resistant strains, designated *erg-2* and *erg-16*, were isolated from a polyene-sensitive strain, *33 erg*$^+$, of *Candida albicans 33 ade*$^-$ following nitrosoguanidine treatment and nystatin enrichment for selection [16] using the method of Molzahn and Woods [12].

Culture media. The following media were used throughout the experiments. Complete medium (YPG): 0.3% yeast extract, 0.5% peptone, 1% glucose. Minimal medium (MM): 1% glucose, 0.5% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, 50 μg/ml adenine, 2 μg/ml biotin, 400 μg/ml thiamine, 2% agar (Difco).

Determination of minimal inhibitory concentrations (MICs). Cells from mid-log phase, shaken cultures were harvested and washed by centrifugation. Cells at a density of 1×10^4/ml were plated on YPG media containing a concentration range of drugs which were freshly prepared in dimethyl sulfoxide. The MICs were determined after incubation for 48 h at 30°C. MIC was defined as the lowest antibiotic concentration that completely inhibited growth under these conditions.

Sterol analysis. For sterol analysis, strains were cultured in MM for 72 h at 30°C on a shaker at 150 rpm, and extracts of non-saponifiable sterols were quantified [24].

Protoplast formation and chitin synthase assay. Four-day-old stock cultures were inoculated at a concentration of 10^6 cells/ml into 2-liter conical flasks containing 400 ml YPG medium and shaken at 150 rpm and 30°C. Cells were collected after attaining 5×10^7 cells/ml. Protoplast formation was carried out as described by Braun and Calderone [1]. The cells were incubated with 2% lyophylized snail enzyme, prepared from *Helix pomatia* in the Department of Microbiology, Attila József University, Szeged, in 1 M mannitol buffered to pH 5.8 with 0.2 M phosphate–0.1 M citrate. Protoplasts were washed (3 times in buffered stabilizer) and then resuspended in 200 mM Tris-hydrochloride, pH 7.5, at 4°C and subjected to four passes in a hand homogenizer.

The lysate was then centrifuged at 100,000 × *g* for 1 h at 4°C in a Beckman L265B ultracentrifuge, and the pellet resuspended in buffer. Preparations were stored overnight at −20°C and used as the enzyme sample. Chitin synthase was assayed according to the method of Ryder and Peberdy [18]. The Tris-hydrochloride buffer system, pH 7.5, employed in this method has been shown to give the same activity with *Saccharomyces cerevisiae* chitin synthase [6] as the imidazole pH 6.5 system, which was first used for this enzyme [4] and in a study of *C. albicans* enzyme [1]. Pro-

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Table 1. Minimal inhibitory concentrations of polyene antibiotics and patterns of cross-resistance in *Candida albicans*.

Strains	Polyene concentration			
	Nystatin (U/ml)	Amphotericin B (μ/ml)	Candicidin (μ/ml)	Pimaricin (μ/ml)
33 erg$^+$	25	1.5	1.5	0.6
erg-2	400	12.0	24.0	10.0
erg-16	400	12.0	24.0	10.0

teolytic activation of chitin synthase preparation was achieved using various concentrations of trypsin (4–8 μg) in Tris-hydrochloride, pH 7.5, containing 1 mM $MgSO_4$ in a total volume of 108 μl to ensure optimal activation of the samples. After incubation for 10 min at 25°C, the digestion was terminated by the addition of soybean trypsin inhibitor (8 μl) to give a concentration double that of trypsin. Duplicate assays were carried out using volumes of 50 μl. In control reactions to determine the active chitin synthase component the trypsin inhibitor was added first.

Chemicals. Nystatin (5,777 U/mg) was purchased from Chinoin (Budapest). Pimaricin was from Gist-Brocades N.U. Mycofarm, Delft. Amphotericin B and candicidin were from Sigma Chemical Co., and other chemicals, of analytical grade, from BDH. UDP-*N*-acetyl-D[*U*-^{14}C]glucosamine (300 μCi/mmol) was purchased from the Radiochemical Centre, Amersham.

Results

Characteristics of strains. The characterization of polyene-sensitive auxotrophs and polyene-resistant mutants has already been reported [14,16]. Thirteen resistant strains were found to have altered sterol composition and could be subdivided into three categories. One group contained nearly as much ergosterol as the parental strain plus an additional sterol, the second had a significantly decreased amount of ergosterol, and the third was strains having no ergosterol.

In the resistant strains, polyene antibiotics induced ion leakage to a smaller degree, as shown by conductometric measurements [16]. Bound Ni^{2+} ions were not liberated by nystatin treatment of resistant cells and their cytoplasm remained unstained after treatment with polyene antibiotics using UO_2^{2+}–Ponceau red stain [14]. The freeze-etched surface of plasmalemma of the *erg-2*-resistant strain showed no detectable changes in structure after treatment with nystatin at a level of the MIC [15]. Two strains from the ergosterol-deficient category were used in subsequent experiments [16].

The MICs of polyene antibiotics for the sensitive strain (*33 erg*$^+$) and the pattern of cross-resistance of nystatin-resistant strains (*erg-2* and *erg-16*) are shown in Table 1. UV-absorption spectra of the ergosterol standard and nonsaponifiable cell extracts (Fig. 1) indicate that the *33 erg*$^+$ strain produces ergosterol, but 24(28)-dehydroergosterol is not present in a detectable amount [24]. A difference was found between the two resistant strains, with the presence of three small peaks between 262 and 269 nm in extracts of the *erg-2* strain. Culture conditions had no effect on the qualitative spectra of the strains. The relative plasma membrane parameter values of the strains that were in exponential growth phase have been determined by electron spin resonance studies by using spin-labeled fatty acid. Sterol mutants had higher order parameter values and thus a greater degree of plasma membrane "rigidity" than their parental strain, as follows: *erg-16* > *erg-2* > *33 erg*$^+$.

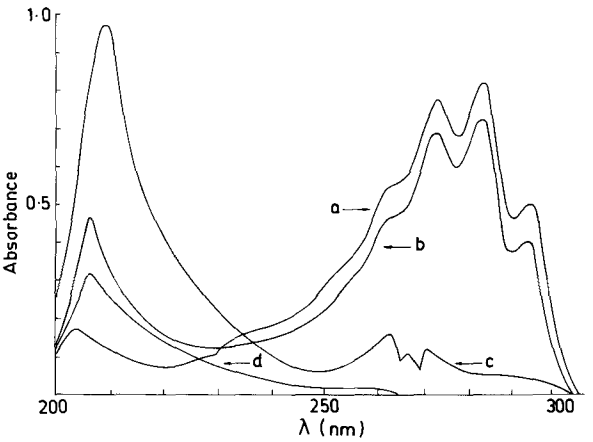

Fig. 1. Ultraviolet absorption spectra of (a) ergosterol standard and sterols extracted from (b) *33 erg*$^+$, (c) *erg-2*, and (d) *erg-16*.

Significant changes were observed in the phospholipid content of sterol mutants as compared with the ergosterol-producing strain (Table 2). The ratio of unsaturated fatty acids to saturated fatty acids in sterol mutants was lower than in their parental strain (M. Pesti, unpublished data). Data on sterol content of these strains will be published elsewhere in detail.

Table 2. Phospholipid content of ergosterol-producing strain of *Candida albicans* and its sterol mutants. Phospholipids are expressed as percentage of total phospholipid phosphorus. The error of the determinations was <5%.

Strains	Percentage of total[a]				
	PC	PE	PI	PS	PA
33 erg$^+$	53.9	16.1	10.6	13.2	6.2
erg-2	33.2	20.7	25.3	5.7	15.3
erg-16	48.0	14.1	23.3	4.3	10.3

[a] PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid.

Table 3. Chitin synthase activities in protoplasts from nystatin-sensitive and -resistant strains of *Candida albicans*. Total activities are expressed as nanomoles GlcNAc per minute per 10^9 protoplasts, and specific activities as nanomoles GlcNAc per minute per milligram protein. Mean values are from four separate experiments, each determination being in duplicate.

Sample	Active		Active + zymogen		Membrane protein (mg/10^9 protoplasts)
	Total	Specific	Total	Specific	
33 erg$^+$	0.55 ± 0.04	0.38 ± 0.04	2.0 ± 0.08	1.35 ± 0.02	1.45 ± 0.09
erg-2	1.17 ± 0.05	0.85 ± 0.03	3.2 ± 0.06	2.40 ± 0.04	1.38 ± 0.11
erg-16	0.74 ± 0.06	0.76 ± 0.04	3.1 ± 0.05	2.05 ± 0.06	1.61 ± 0.14

Chitin synthase activity. Protoplast preparations were obtained after a 1-h digestion of cells with snail enzyme, which gave 100% conversion. The values of chitin synthase activity in the active form and zymogen form (after trypsin digestion) are given in Table 3. The total activity, active plus zymogen, is the optimum value determined following trypsin digestions. The chitin synthase specific activity, both active and zymogenic, is higher in the sterol mutants than in the parental strain. In the case of stationary-phase cells, where budding has virtually ceased, the active component of the sensitive and resistant strains was found to be at the same low specific acitvity (0.12 ± 0.02 nmol GlcNAc/min/mg protein), whereas the zymogenic component still showed greater susceptibility to trypsin digestion in the resistant strains (*erg-2* = 0.46 ± 0.02 and *erg-16* = 0.42 ± 0.04 nmol GlcNAc/min/mg protein) than in the sensitive one (0.31 ± 0.03 nmol Glc/min/mg protein).

Discussion

Several studies have shown that altered lipid content of the cell membrane in fungi has a marked consequence on cellular processes. In *Candida albicans* sterol mutants having lower ergosterol content, a decreased rate and level of accumulation of certain amino acids has been found [20], and changes in inositol-containing lipids of *Neurospora crassa* plasmalemma may have been the cause of the observed major effects on cell wall synthesis [7]. A preliminary investigation of carbohydrate assimilation by *C. albicans* nystatin-resistant strains showed significant reductions in comparison to the sensitive strain [16]. It is significant that sterol incorporation into membranes has been shown to increase order and rigidity of both artificial membrane systems [2,13] and the cell membrane of *C. albicans* [10]. It has also been proved that sterols could regulate the plasma membrane lipid composition by adaptive alteration in living organisms [19].

This type of change in cell surface phenomena, possibly due to the absence of ergosterol in the plasmalemma, prompted the investigation of chitin synthase activities of polyene-sensitive and -resistant cells. This enzyme has been shown in yeast to be in a zymogenic form, which is activated during budding [3,4]. The transformation to the active chitin synthase may be brought about by an endogenous protease, proteinase B [4,22], itself under the control of a proteinaceous inhibitor [21]. This proteinase B has also been shown to inactivate the active chitin synthase at a slower rate than that activation [8] which would complete the regulation of the localized deposition of chitin in the bud wall. The higher total and specific chitin synthase activities, both active and zymogenic, observed in the *C. albicans* sterol mutants may be explained in relation to the regulatory mechanism controlling chitin synthase activity. In the changed membrane environment, the protease functions of activation and inactivation may be altered in such a way that there is greater chitin synthase activity existing at the population level. The observed increase in chitin synthase activity may also be interpreted in the following terms: the greater rigidity of ergosterol-deficient membranes resulted in the increased accessibility of activating factor or trypsin to the zymogenic form of chitin synthase and so increased the number of active enzymes by which both the total and the specific activity were enhanced. The conformation of the enzyme changed favorably as a consequence of the complex alterations of plasmalemma lipid composition to allow greater activity in sterol mutants. The purified chitin synthase from *S. cerevisiae* has been shown to have a requirement for certain phospholipids [6], and in *C. albicans* the plasma membrane–bound mannan synthetase is influenced by its lipid environment [11]. Certain lipid molecules in model membrane systems, "boundary lipids" lying adjacent to protein components, have been shown to be tightly associated with membrane enzyme molecules [23], and this type of interrelationship of lipid and protein would obviously be of fundamental importance to any integral membrane enzyme.

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