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Interactions of oxidosqualene cyclase (Erg7p) with 3-keto reductase (Erg27p) and other enzymes of sterol biosynthesis in yeast

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SUMMARY

In *Saccharomyces cerevisiae* and *Candida albicans,* two enzymes of the ergosterol biosynthetic pathway, oxidosqualene cyclase (Erg7p) and 3-keto reductase (Erg27p) interact such that loss of the 3-keto reductase also results in a concomitant loss of activity of the upstream oxidosqualene cyclase. This interaction wherein Erg27p has a stabilizing effect on Erg7p was examined to determine whether Erg7p reciprocally has a protective effect on Erg27p. To this aim, three yeast strains each lacking the ERG7 gene were tested for 3-ketoreductase activity by incubating either cells or cell homogenates with unlabeled and radiolabeled 3-ketosteroids. In these experiments, the ketone substrates were effectively reduced to the corresponding alcohols, providing definitive evidence that oxidosqualene cyclase is not required for the 3-ketoreductase activity. This suggests that, in *S. cerevisiae*, the protective relationship between the 3-keto reductase (Erg27p) and oxidosqualene cyclase (Erg7p) is not reciprocal. However, the absence of the Erg7p, appears to affect other enzymes of sterol biosynthesis downstream of lanosterol formation. Following incubation with radiolabeled and non-radiolabeled 3-ketosteroids we detected differences in hydroxysteroid accumulation and ergosterol production between wild-type and *ERG7* mutant strains. We suggest that oxidosqualene cyclase affects Erg25p (C-4 sterol oxidase) and/or Erg26p (C-3 sterol dehydrogenase/C-4 decarboxylase), two enzymes that, in conjunction with Erg27p are involved in C-4 sterol demethylation.

1. Introduction

Sterol biosynthesis (Fig. 1) is a complex pathway in which the enzyme oxidosqualene cyclase (OSC) connects two sharply different parts: (i) the “assembly section”, a sort of assembly line, which gathers all the components required to form the open triterpene 2,3-oxidosqualene, and (ii) a “tailoring section”, which remodels the steroid-intermediates to produce the final product (cholesterol, ergosterol or phytosterols, in animal, fungal, or plant cells, respectively). For the cyclization of oxidosqualene, the critical task of the OSC enzyme is to properly fold the open triterpene oxidosqualene to generate the four membered steroid ring. Despite its structurally

critical role, oxidosqualene cyclase is not considered a regulatory enzyme in sterol biosynthesis, as are HMGCoA-reductase [1,2] and squalene epoxidase [3,4]. In mammals, OSC indirectly plays a role in lipid regulatory processes owing to its ability to cyclize dioxidosqualene to 24,25-epoxylanosterol, a precursor of 24,25-epoxycholesterol which is one of the most potent naturally occurring ligand activators of the liver X receptor [5,6,7,8]. Thus, in mammals, OSC appears to interact via dioxidosqualene formation with an impressive series of regulatory processes that control lipid metabolism [8]. In yeast, interactions involving OSC are less defined although recent studies have demonstrated that OSC is not a solitary protein. The OSC enzyme directly interacts with another enzyme of sterol biosynthesis, the 3-ketosterol reductase (Erg27p), an enzyme involved in C-4 demethylation of intermediates of ergosterol biosynthesis [9,10]. In yeast cells in which the ERG27 gene is deleted, oxidosqualene cyclase (Erg7p) is inactive and thus the "tailoring section" of sterol biosynthesis, which includes the 3-ketoreductase-catalyzed step, appears to affect the step which generates the steroid nucleus. The protective effect of Erg7p by Erg27p, is only one example of protein-protein interactions in sterol biosynthesis. Mo et al. suggested that all the members of the C-4 demethylation apparatus, Erg25p, Erg26p, Erg27p and Erg28p, interact as a complex [11]. More recently, a split-ubiquitin two hybrid analysis of the ergosterol biosynthetic pathway predicted several protein-protein interactions, some of which included OSC, suggesting that the entire pathway may be organized into an enzyme complex [12].

In the present study, we further examined the interaction between oxidosqualene cyclase (Erg7p) and 3-keto reductase (Erg27p) in yeast in order to establish whether the protective action of Erg27p toward Erg7p is reciprocal. Additionally, we investigated possible interactions of Erg7p with other enzymes of the post-squalene section of sterol biosynthesis by following the metabolic fate of radiolabeled and non-radiolabeled substrates such as 3-ketosteroids in yeast strains lacking the ERG7 gene. Our data showed that the interaction between oxidosqualene

cyclase and 3-keto reductase is not reciprocal, while the absence of Erg7p affects other enzymes of the C-4 demethylation complex.

2. Materials and methods

2.1 *S. cerevisiae strains and growth conditions*

The genotypes of strains used in this study are listed in Table 1. The *ERG7* allele in strain STY2 was disrupted by using one-step PCR deletion-disruption [13]. The primers F erg7del (5'-GCCTCTCCAGTAATGTACTGCTGTGCCCAATAACCTTACCAATAATCGTCG Tggcgggtgtcggggctggc-3') and R erg7del (5'-GCGTATGTGTTTCATATGCCCTGCTGTACATA CCTAATGCCTTAATAGGGttgccgatttcggcctattg-3') were used to generate a 1.5-kilobase (kb) *erg7* recombinogenic DNA fragment containing *HIS3* as a selectable marker and using pRS303 [14] vector as template. Bases in lowercase correspond to conserved regions flanking the HIS3 gene in the pRS303 vector, and bases in uppercase refer to upstream and downstream *ERG7* sequence. The *ERG7* knockout was confirmed by the sterol GC profile as well as by diagnostic PCR using a primer sequence up-stream of the *ERG7* deleted sequence as well as a primer in the HIS3 gene. Strain STY32 was constructed by integrating a 2.3-kb *HindIII-SpeI ERG7* DNA fragment into the multiple-cloning site of the high-copy plasmid pRS426Gal (to generate the plasmid pST8) and subsequently transforming STY2 (*erg7*).

All plasmids were maintained in *Escherichia coli* DH5α cultured in LB medium supplemented with ampicillin (100 μg/ml). PCR reactions were performed on a Thermocycler apparatus using the Promega *Taq* polymerase kit.

Wild-type strains were grown to early stationary phase in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C. Mutants disrupted for either *ERG7* or *ERG27* were supplemented with ergosterol (0.02 mg/ml). SMY8, an *erg7* mutant which additionally is a heme auxotroph (*hem1::TRP1*) was grown aerobically in media supplemented with hemin (0.013 mg/ml). The

yeast *erg7* mutant STY2, that contains *upc2-1*, was grown aerobically as this allele allows for aerobic sterol uptake [15]. The *ERG7* deletant strain GDY7 and the *ERG27* deletant strain SDG115, were also able to grow under aerobic conditions, with sterol supplementation and were therefore grown aerobically. The strains STY32 and SMY8[pSM61.21], overexpressing *ERG7* under control of GAL1 promoter, were grown in the presence of galactose. Transformants were grown on complete synthetic-dropout media containing 0.67% yeast nitrogen base, 2% glucose/galactose, 0.2% aminoacids and 0.5% ammonium sulphate, supplemented with ergosterol (0.02 mg/ml).

2.2 Chemicals

Buffers, culture media, cholesterol, ergosterol and bovine albumin, used as a standard for protein determination, were purchased from Sigma-Aldrich (Italy).

2.3 Radiolabeled substrates

Radiolabeled (R,S)-[2-^{14}C]mevalonic acid (2.04 GBq/mmol) and [2-^{14}C]acetic acid (2.04 GBq/ mmol) were purchased from Amersham Pharmacia Biotech (UK).

[^{14}C](3S)-2,3-oxidosqualene was prepared as previously reported [16]. [^{14}C]lanosterol was prepared according to a protocol described for preparing radiolabeled oxidosqualene [16], with modifications. Briefly, 0.85 mL of a pig liver S10 preparation (25 mg protein) was incubated for 3 h at 37°C with (R,S)-[2-^{14}C]mevalonic acid (0.5 µCi, 2.04 GBq/mmol) in 0.1 M Tris buffer (pH 7.4, final volume 1 mL) containing 0.1 mM Ketoconazole. The incubation mixture also contained Tween 80 (0.1 mg/mL), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 30 mM nicotinamide, 1 mM ATP and a NADPH generating system (1 mM $NADP^+$, 3 mM glucose-6-phosphate and 6 units of G-6-P dehydrogenase). The addition of ATP and the NADPH generating system was repeated at the end of the first and second h. The reaction was stopped by adding 1 mL of methanolic KOH (15% w/v) and lipids were saponified at 80°C for 30 min. The nonsaponifiable lipids were then extracted three times with 2 mL of petroleum ether and separated by TLC on silica gel plates (20x20 cm x 0.25 mm) using cyclohexane/ethyl acetate (85:15; v/v) as the

developing system. The radioactive band corresponding to lanosterol was scraped off and eluted with dichloromethane. The [^{14}C]lanosterol extract was then dried under nitrogen and dissolved in benzene. The average yield of the preparation was 0.035 μCi.

The radiolabeled substrate for the 3-ketoreductase activity assay was prepared by incubating a homogenate from an *ERG27* knockout strain, SDG115, with [^{14}C]lanosterol. Briefly, 0.85 mL of a SDG115 homogenate (40-50 mg of protein) was incubated with [^{14}C]lanosterol (0.1 μCi) in the presence of Tween 80 (0.1 mg/mL), 1 mM ATP, 1 mM NAD^+ and a NADPH generating system (described above). The incubation lasted for 16 h at 30°C with vigorous shaking. Addition of the ATP and NADPH generating system was repeated at each h for the first four h. The reaction was stopped by adding 1 mL of methanolic KOH (15% w/v), and lipids were saponified at 80°C for 30 min. The nonsaponifiable lipids were extracted three times with 2 mL of petroleum ether and separated by TLC on silica gel plates (20x20 cm x 0.25 mm) using cyclohexane/ethyl acetate (85:15; v/v). The two main radioactive chromatographic bands, designated 3-ketosteroid 1 and 2 (Rf 0.75 and 0.54, respectively) were scraped off and eluted with dichloromethane. The extracts were dried under nitrogen and each ketosteroid was dissolved in benzene.

The ketonic nature of the compounds was established by treatment with $NaBH_4$. An aliquot ($9x10^{-4}$ μCi) of each radiolabeled compound was then incubated with 5 mg of $NaBH_4$ in 1 mL of ethanol for 30 min with magnetic stirring. The reaction was stopped by adding 1 mL of water, and the reaction mixture was extracted twice with 1 mL of petroleum ether and separated on silica gel plates using cyclohexane/ethyl acetate (85:15; v/v). The transformation yield for both 3-ketosteroid substrates, 1 and 2**,** was >90 % as judged by the distribution of radioactivity following chemical reduction.

2.4 Sterol accumulation in an erg7 strain grown with different sterols

The GDY7 (*erg7*) strain was grown overnight in YPD media supplemented with ergosterol, cholesterol, lanosterol or ergosta-7,22-diene-3-one (0.02 mg/mL). Cells were harvested and

saponified for 1 h at 80°C in ethanolic KOH (25% w/v). The nonsaponifiable lipids were extracted once with one volume of n-heptane and analyzed by GC-MS. Gas-chromatographic-mass spectrometric analyses to determine sterol profiles was carried out as described [17].

2.5 Incorporation of [2-^{14}C]acetate into cells

Sterol biosynthesis in whole yeast cells was followed by incorporation of [2-^{14}C]acetate into nonsaponifiable lipids as described previously [18]. Briefly, washed cells ($1x10^8$ cells) were resuspended in 1 mL of fresh media, incubated with 0.1 µCi of [2-^{14}C]acetate and shaken for 3 h at 30°C. Cells were then saponified in 1 mL of methanolic KOH (15% w/v) for 30 min at 80°C. Nonsaponifiable lipids were extracted twice with 1.5 mL of petroleum ether and separated on TLC plates (20x20 cm x 0.25 mm) using cyclohexane/ethyl acetate (85:15; v/v). Squalene, oxidosqualene, dioxidosqualene, lanosterol and ergosterol were used as references. Radioactivity in separated bands was determined using a System 200 Imaging Scanner (Hewlett-Packard, Palo Alto, CA, USA).

2.6 Erg7 and Erg27 enzyme assays with radiolabeled substrates

Cell-free homogenates were prepared as described [19]. After lysis of the yeast cell wall with lyticase, spheroplasts were homogenized with a Potter device. Proteins in the homogenate were quantified using the SIGMA Protein Assay Kit [20] with bovine serum albumin as a standard. The enzyme assay for Erg7p has been described previously [19]. Cell homogenates (1.5 mg protein) were incubated for 1 h at 35°C with [^{14}C](3S)-2,3-oxidosqualene ($4.5x10^{-4}$ µCi) and the enzymatic reaction resulting in lanosterol was terminated by adding 1 mL of methanolic KOH (15% w/v). Lipids were saponified at 80°C for 30 min and nonsaponifiable lipids were extracted with petroleum ether. Extracts were then spotted on TLC plates using cyclohexane/ethyl acetate (85:15; v/v) as the developing solvent. The conversion of labeled oxidosqualene to labeled lanosterol was quantified with a System 200 Imaging Scanner (Hewlett-Packard, Palo Alto, CA, USA).

The 3-ketoreductase (Erg27p) activity was assayed by a novel procedure in which a radiolabeled 3-ketosteroid intermediate of ergosterol biosynthesis was used as a substrate. The yeast cell homogenates (3 mg protein) were incubated with $7x10^{-4}$ μCi of either [^{14}C]3-ketosteroid 1 or 2 in the presence of Tween 80 (0.1 mg/mL), 1 mM ATP and an NADPH generating system (indicated above) in 0.1 M Tris (pH 7.4, final volume 0.5 mL) buffer containing 5 mM $MgCl_2$, 2 mM $MnCl_2$, 30 mM nicotinamide, for 3 h at 30°C with vigorous shaking. The reaction was terminated by adding 1 mL of methanolic KOH (15% w/v), and lipids were saponified at 80°C for 30 min. The nonsaponifiable lipids were extracted twice with 1.5 mL of petroleum ether and spotted on TLC plates using cyclohexane/ethyl acetate (85:15; v/v) as the developing solvent. The conversion of labeled 3-ketosteroids to labeled reaction products was quantified with a System 200 Imaging Scanner (Hewlett-Packard, Palo Alto, CA, USA). Enzyme activity was expressed as a ratio of products formed/starting compound. The actual enzyme specific activity (amount of product formed/time/mg protein) could not be calculated as the specific radioactivity of the radiolabeled substrates could not be determined.

3. Results

3.1 Incubation with radiolabeled acetate and oxidosqualene

Cells of *ERG7* deletant strains were incubated with radiolabeled acetate to assess possible differences in sterol profiles resulting from different strain backgrounds. As expected, all *erg7* strains accumulate labeled oxido- and dioxidosqualene with the latter predominanting. In two of them, STY2 and SMY8, dioxidosqualene accounts for more than 90% of the non saponifiable lipids (Table 2). The absence of oxidosqualene cyclase activity in *erg7* strains was confirmed by incubation of cell homogenates with radiolabeled oxidosqualene (Table 3). Accumulation of radioactivity into end-product ergosterol in wt and *ERG7*-overexpressing strains STY32 and SMY8[pSM61.21] verified the integrity of the post-squalene pathway. Surprisingly, in the STY32 strain oxido- and dioxidosqualene also accumulate. However, oxidosqualene cyclase

activity detected in the homogenate prepared from this strain is comparable to that measured in both wt and the other *ERG7*-overexpressing strains (Table 3).

3.2 Sterol accumulation in an erg7 strain grown with ergosta-7,22-diene-3-one

We initially approached the question of the effect of the absence of Erg7p on 3-ketosterol reductase (Erg27p) activity by growing an *ERG7* deletant strain (GDY7) with ergosta-7,22-diene-3-one, a substrate of Erg27p. In parallel, cells were grown separately with cholesterol, ergosterol or lanosterol. As expected, cells accumulated oxy- and dioxysqualene regardless of the sterol added to the growth medium. In cells incubated with ergosta-7,22-diene-3-one, 75% of this sterone was converted to ergosta-7,22-diene-3-ol, clearly indicating that the 3-ketoreductase enzyme was active in the absence of oxidosqualene cyclase. The observation that more than half of the total sterol was end-product ergosterol in the same ketone-fed cells clearly suggests that not only was ergosta-7,22-diene-3-one actively reduced to the corresponding alcohol, but also the latter was converted to end-product ergosterol (Table 4).

3.3 Preparation and characterization of the radiolabeled physiological substrates of 3-ketosterol reductase.

A problem we initially encountered was the preparation of a proper radiolabeled substrate for determining 3-ketosterol reductase activity as no radiolabeled 3-ketosteroid is commercialy available. Since our aim was to test Erg27p activity with authentic keto-intermediates of ergosterol biosynthesis, we prepared biological relevant substrates using a yeast strain lacking *ERG27*. However, since an *erg27* strain is unable to cyclize oxidosqualene [21,9], we could not generate ketosteroid intermediates by incubating cells with radiolabeled mevalonate or acetate. Therefore, we employed a SDG115 (*erg27*) cell-free system incubated with radiolabeled lanosterol, an intermediate downstream of oxidosqualene cyclase. Incubation with labeled lanosterol gave two steroids designated ketosteroid 1 and 2 (Fig. 2), which were identified as keto compounds by treatment with $NaBH_4$ (Fig. 3).

Both radiolabeled compounds gave more polar derivatives on a TLC plate as expected when alcohol derivatives are formed from treating ketone compounds with $NaBH_4$. From 3-ketosteroid compound 1, two more polar compounds (Rf 0.62 and 0.39) were formed, presumably corresponding to α- and β-hydroxy derivatives resulting from the reduction of the keto group of the starting radiolabeled compound. The alpha and beta configurations at C-3 could be attributed by both chemical and biochemical observations. In steroids, due to the chair conformation of ring A, the alpha and beta bonds at C-3 are oriented axial and equatorial, respectively. Since a bulky hydroxyl-substituent at C-3 will preferably adopt the equatorial orientation, the beta configuration should predominate in the alpha/beta mixture that results from the reduction of the corresponding 3-keto compound. The compound bearing the beta-equatorial hydroxyl group should be more polar (with a lower Rf value on TLC silica gel plates), as the polar substituent is more exposed to the solvent than the alpha-axial hydroxyl group. We therefore attributed the beta configuration to the more abundant and more polar compound reported in Fig. 3. This attribution was confirmed by comparing the chemical (alpha/beta-generating) and biochemical (solely beta-generating) reduction of the intermediate ketosteroid 1 (Fig. 4A). The 3-ketosteroid compound 2, treated with $NaBH_4$, gave a unique even more polar compound (Rf 0.27), which was presumably a mixture of α- and β –hydroxy derivatives indistinguishable by TLC.

To further characterize these radiolabeled products, a small amount (approx. 0.05 mg) of non-radioactive 3-ketosteroids 1 and 2 was prepared by incubating the cell homogenate with non radioactive lanosterol under similar conditions. Chromatographic bands corresponding to 3-ketosteroid 1 and 2 were scraped off, extracted, and analyzed by GC-MS: 3-ketosteroid 1 gave two major peaks identified as 4-methylzymosterone and 4-methylfecosterone + 4-methylepisterone (both in one huge GLC peak); 3-ketosteroid 2 gave three peaks identified as zymosterone, ergosta-7,22-diene-3-one and episterone. Some of these 3-ketosteroids have been previously identified by GLC-MS in another *erg27* strain (SDG110) [21]. As expected, the main

structural difference between the mixtures referred to as ketosteroid 1 and ketosteroid 2 is the presence of a methyl group at C-4, that explains the different TLC behaviour: the less polar methyl-bearing compounds ("ketosteroid 1") have a higher Rf value on TLC than "ketosteroid 2" (Fig. 2).

Thus, GLC-MS analysis revealed that all unlabeled compounds co-chromatographing on TLC with radiolabeled ketosteroid 1 and 2 were ketone compounds. This confirmed that the radiolabeled compounds called ketosteroid 1 and 2 are actually keto intermediates of ergosterol biosynthesis and can be properly used as radiolabeled substrates to test the activity of 3-ketosterol reductase.

3.4 Incubation of homogenates from cells lacking the ERG7 gene with radiolabeled 3-ketosteroids

To evaluate the effect of the absence of oxidosqualene cyclase (Erg7p) on 3-ketosterol reductase (Erg27p) activity, three yeast strains lacking the ERG7 gene were compared to a wild-type strain and strains overexpressing *ERG7*. Homogenates from all strains were incubated aerobically with radiolabeled 3-ketosteroids 1 and 2 under conditions indicated in Materials and Methods. In both *erg7* mutant and *ERG7* (wild-type and overexpressing) strains, sterone substrates 1 and 2 were effectively transformed into more polar compounds, which were likely to be the corresponding alcohols (Fig. 4 and 5; Table 5 and 6). The alcoholic nature of the products formed was inferred by comparing their chromatographic behaviour with those of compounds obtained by treating radiolabeled sterones 1 and 2 with $NaBH_4$. The biological reduction gave compounds co-chromatographing with those obtained from chemical reduction. Particularly, enzymatic reduction of sterone 1 resulted in a compound that co-chromatographed with the more polar (beta-hydroxy substituent) which resulted from chemical reduction.

In experiments with radiolabeled 3-ketosteroid 1 as the substrate, we observed a strong difference between *erg7* and *ERG7* strains. In the homogenates from the *erg7* strains, the 3-hydroxy product of the 3-ketoreductase activity was no longer transformed to the end-product

ergosterol (Fig. 4; Table 5), whereas in both wild-type and overexpressing strains, ergosterol was synthesized. To reinforce the results and conclusions obtained from radiolabeling experiments and confirm the chemical identity of the products formed by enzymatic reduction of ketosteroid 1, parallel experiments with non radioactive ketosteroid 1 were performed. GLC analysis of the chromatographic band recovered from the experiments with "ketosteroid 1" revealed the presence of a mixture of methylzymosterol and methylfecosterol. With radiolabeled 3-ketosteroid 2, the difference between *erg7* and *ERG7* strains could not be determined as the transformation product of the sterone substrate co-localized with ergosterol on TLC plates (Fig. 5; Table 6).

4. Discussion

In the present study, we demonstrated that the strong interaction between Erg7p and Erg27p in yeast is not reciprocal in that the requirement of Erg27p for oxidosqualene cyclase (Erg7p) activity is absolute [8], whereas Erg27p activity is independent of the Erg7p: the 3-ketosterol reductase (Erg27p) in strains lacking Erg7p is as active as in the wild-type strain.

The absence of oxidosqualene cyclase, however, seems to greatly affect the functionality of several other enzymes in the post-squalene biosynthetic pathway. The following evidence supports this conclusion: when homogenates prepared from *erg7* strains were incubated with one of the radiolabeled sterone substrates (3-ketosteroid 1), the post-squalene enzyme apparatus was unable to synthesize end-product ergosterol. Conversely, all control strains, either wild-type or *ERG7*-overexpressing strains, were able to convert radiolabeled ketosteroid 1 to ergosterol. These experiments do not allow us to unambiguously identify the enzyme(s) affected by the absence of oxidosqualene cyclase but suggest that the inability to synthesize oxidosqualene results in an inability to convert downstream sterol products. However, comparison between experiments with non radioactive ergosta-7,22,diene-3-one (section 3.2) and experiments with both radiolabeled and non-radiolabeled ketosteroid 1 strongly narrows the possible enzymes

putatively affected by the absence of Erg7p. While the *ERG7* deletant strains cannot carry out the transformation of ketosteroid 1 (actually, a mixture of monomethylsterones) beyond the reduction to the corresponding alcohol(s), one of these strains, incubated with nonradioactive ergosta-7,22,diene-3-one (a 4,4-desmethylsterone), not only reduces it to the hydroxy derivative but also converts the sterol intermediate to ergosterol. These results suggest that Erg6p, Erg3p and Erg4p, the enzymes required to bridge the gap that separates ergosta-7,22-diene-3-ol from ergosterol, are active in an *erg7* strain. Thus, the enzymatic block that prevents radiolabeled ketosteroid 1 from further downstream conversions, beyond its reduction to the alcohol, seems to be the presence of the methyl group at C-4.

Therefore, we suggest that the inability of the *erg7* deletant strains to form ergosterol from monomethyl intermediates of sterol biosynthesis depends on the loss of funtionality of the C-4 demethylation complex in the absence of Erg7p. This complex is made up of three enzymes, Erg25p, Erg26p, and Erg27p, which are involved in the removal of the C-4 methyl groups from 4,4-dimethylzymosterol. This complex requires a transmembrane scaffold protein, Erg28p, which anchors the enzymes to the endoplasmic reticulum. The evidence suggested in this study indicates that Erg27 activity does not require the presence of the Erg7p whereas either Erg25p (C-4 sterol oxidase), Erg26p (C-3 sterol dehydrogenase/C-4 decarboxylase) or both may be negatively affected by the absence of Erg7p. We cannot exclude the possibility that deletion of *ERG7* has a negative effect on the ability of Erg28p to function as a scaffold protein of the C-4 demethylation complex.

In a recent study using the split-ubiquitin membrane protein yeast two-hybrid system, an interaction between Erg7p and three components of the complex, Erg25p, Erg27p and Erg28p, was predicted [12]. Our previous [9,10] and present results confirm this prediction, suggesting a subtle bidirectional relationship between Erg7p and the C-4 demethylation apparatus.

The bidirectional control mechanism between the demethylation complex and oxidosqualene cyclase can be depicted as follows: the complex, formed by Erg25p, Erg26p, Erg27p and

Erg28p, controls the functionality of oxidosqualene cyclase through a direct chaperone-like action of Erg27p toward Erg7p, and additionally, oxidosqualene cyclase controls the functionality of the complex by interacting with component(s) other than Erg27p. Whether the loss of Erg7p affects the stability of Erg25p, Erg26p or Erg28p remains to be established and will require future studies.

These findings lead us to speculate regarding possible novel roles of oxidosqualene cyclase(s) in the regulation of sterol biosynthesis. The C-4 demethylation complex catalyzes the reactions of the highest oxygen-consuming step of sterol biosynthesis, and is thought to play a specific role in oxygen sensing in eukaryotes [22]. Unravelling the interaction between enzymes of the C-4 demethylation complex and oxidosqualene cyclase (as well as other enzymes of sterol biosynthesis) may help to clarify mechanisms of adaptation to low oxygen levels, a condition that yeast cells share with many human cancer cells which are able to survive in oxygen starved tumors. An additional reason to further explore the interaction between oxidosqualene cyclase and the C-4 demethylation complex lies in the close link between the deficiency of one of the enzymes of this complex and the CHILD syndrome, a severe disorder of cholesterol biosynthesis [23].

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Figure Legends

Fig. 1. The *S. cerevisiae* ergosterol biosynthetic pathway from squalene to the end-product ergosterol. Compounds in bold match to the mixture of 3-ketosteroid 1, compounds in bold and underlined to the mixture of 3-ketosteroid 2. Genes in the pathway blocked in *erg7* strains are designated in grey.

Fig. 2. Synthesis of 3-ketosteroids. Radiochromatogram of non-saponifiable lipids extracted from cell homogenates of strain SDG115 (*erg27*) incubated with radiolabeled lanosterol. Lanosterol (peak a) was converted into 3-ketosteroid 1 (peak c) and 3-ketosteroid 2 (peak b).

Fig. 3. Chemical reduction of [^{14}C]3-ketosteroid 1 with $NaBH_4$. Radiochromatogram of the compounds resulting from reduction of 3-ketosteroid 1 with excess $NaBH_4$ (see Exp. Section). Peak c is 3-ketosteroid 1, peak a is the β-hydroxy derivative of 3-ketosteroid 1, and peak b is the α-hydroxy derivative.

Fig. 4. Incubation of homogenates from the *erg7* strain SMY8 (Radiochromatogram A) and the *ERG7*-overexpressing strain SMY8[pSM61.21] (Radiochromatogram B) with [^{14}C]3-ketosteroid 1. In A, the 3-ketosteroid 1 (peak b) was transformed only into the 3-hydroxy derivative (peak a), whereas in B, the 3-ketosteroid 1 (peak c) was transformed into both 3-hydroxy derivative (peak b) and ergosterol (peak a), the end-product of the pathway (see Results for details).

Fig. 5. Radiochromatogram of non-saponifiable lipids extracted from cell homogenates of the *ERG7*-overexpressing strain SMY8[pSM61.21] incubated with [^{14}C]3-ketosteroid 2. 3-ketosteroid 2 (peak b) was converted into 3-hydroxyderivate (peak a), which was indistinguishable from ergosterol on TLC.

Table 1

S. cerevisiae strains and plasmids used in this study. Growth conditions are reported in the Materials and methods section.

Strain	Genotype
SDG115	*MATa, ade5, his7, leu2-3,112, ura3-52, erg27Δ::URA3*
SCY876	*MATα, upc2-1, hap1Ty, ura3, his3, leu2, trp1*
STY2	*MATα, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg7Δ::HIS3*
STY32	*MATα, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg7Δ::HIS3*[pST8](*ERG7*)
FY1679-06C	*MATα, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, GAL2*
GDY7	*MATα, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, GAL2, erg7Δ::KANMX4*
SMY8	*MATa, erg7::HIS3, hem1::TRP1, ura3-52, trp1-Δ63, leu2-3,112, his3Δ200, ade2, Gal*$^+$
SMY8[pSM61.21]	*MATa, erg7::HIS3, hem1::TRP1, ura3-52, trp1-Δ63, leu2::pSM61.21*(*ERG7*), *his3Δ200, ade2, Gal*$^+$
Plasmid	
pST8	pRS426GAL [1] *ERG7* HIndIII-SpeI
pSM61.21	pRS305GAL *ERG7* NotI-SalI [2]

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Table 2

Incorporation of [^{14}C]acetate into nonsaponifiable lipid fraction

Strain	% total radioactivity incorporated					
	Squalene	Oxido squalene	Dioxido squalene	Lanosterol	4-monomethyl sterols*	Ergosterol
SCY876 (wt)	29.38	17.94	1.61	3.34	3.33	44.40
STY2 (*erg7*)	1.05	8.87	90.08	0.00	0.00	0.00
STY32 (*erg7*/p*ERG7*)	5.51	12.68	45.04	1.77	1.23	33.77
FY1679-06C (wt)	16.48	5.49	1.26	9.03	2.21	65.53
GDY7 (*erg7*)	12.56	27.27	60.17	0.00	0.00	0.00
SMY8 (*erg7*)	2.46	7.10	90.44	0.00	0.00	0.00
SMY8 [pSM61.21] (*erg7*/p*ERG7*)	12.97	3.11	2.78	5.79	3.86	71.49

* TLC bands with Rf values ranging from lanosterol to ergosterol

Results are the means of two separated experiments with duplicate incubations, each. The maximum deviations from the mean were less then 10%.

Table 3

Erg7p specific activity in homogenates

Strain	Erg7p specific activity (nmol h^{-1} mg^{-1})
SCY876 (wt)	0.91
STY2 (*erg7*)	0.00
STY32 (*erg7*/p*ERG7*)	1.00
FY1679-06C (wt)	1.63
GDY7 (*erg7*)	0.00
SMY8 (*erg7*)	0.00
SMY8 [pSM61.21] (*erg7*/p*ERG7*)	1.75

Results are the means of at least two separated experiments with duplicate incubations, each. The maximum deviations from the mean were less then 10%.

Table 4

GC profiles of GDY7 (*erg7*) strain grown in presence of various sterols

Media YPAD	% nonsaponifiable lipids							
	Oxido squalene	Dioxido squalene	Lanosterol	Lanost-8-en-3-ol	Ergosta-7,22-diene-3-one	Ergosta-7,22-diene-3-ol	Cholesterol	Ergosterol
+ergosterol	9.48	24.89	0.00	0.00	0.00	0.00	0.00	65.63
+cholesterol	20.98	6.50	0.00	0.00	0.00	0.00	72.52	0.00
+ergosta-7,22-diene-3-one	9.39	20.98	0.00	0.00	7.40	23.12	0.00	39.11
+lanosterol	58.54	23.56	5.40	12.50	0.00	0.00	0.00	0.00

Table 5

Incubation of homogenates with [^{14}C]3-ketosteroid 1

Strain	% radioactivity in nonsaponifiable lipids		
	3-ketosteroid 1	3-hydroxysteroid 1	Ergosterol
SCY876 (wt)	47.85	32.95	19.20
STY2 (*erg7*)	47.47	48.50	4.03
STY32 (*erg7*/p*ERG7*)	42.03	20.77	37.20
FY1679-06C (wt)	37.80	53.48	8.72
GDY7 (*erg7*)	42.02	55.45	2.53
SMY8(*erg7*)	34.99	60.33	4.68
SMY8 [pSM61.21] (*erg7*/p*ERG7*)	40.85	34.67	24.48

Results are the means of at least two separated experiments with duplicate incubations, each. The maximum deviations from the mean were less then 10%.

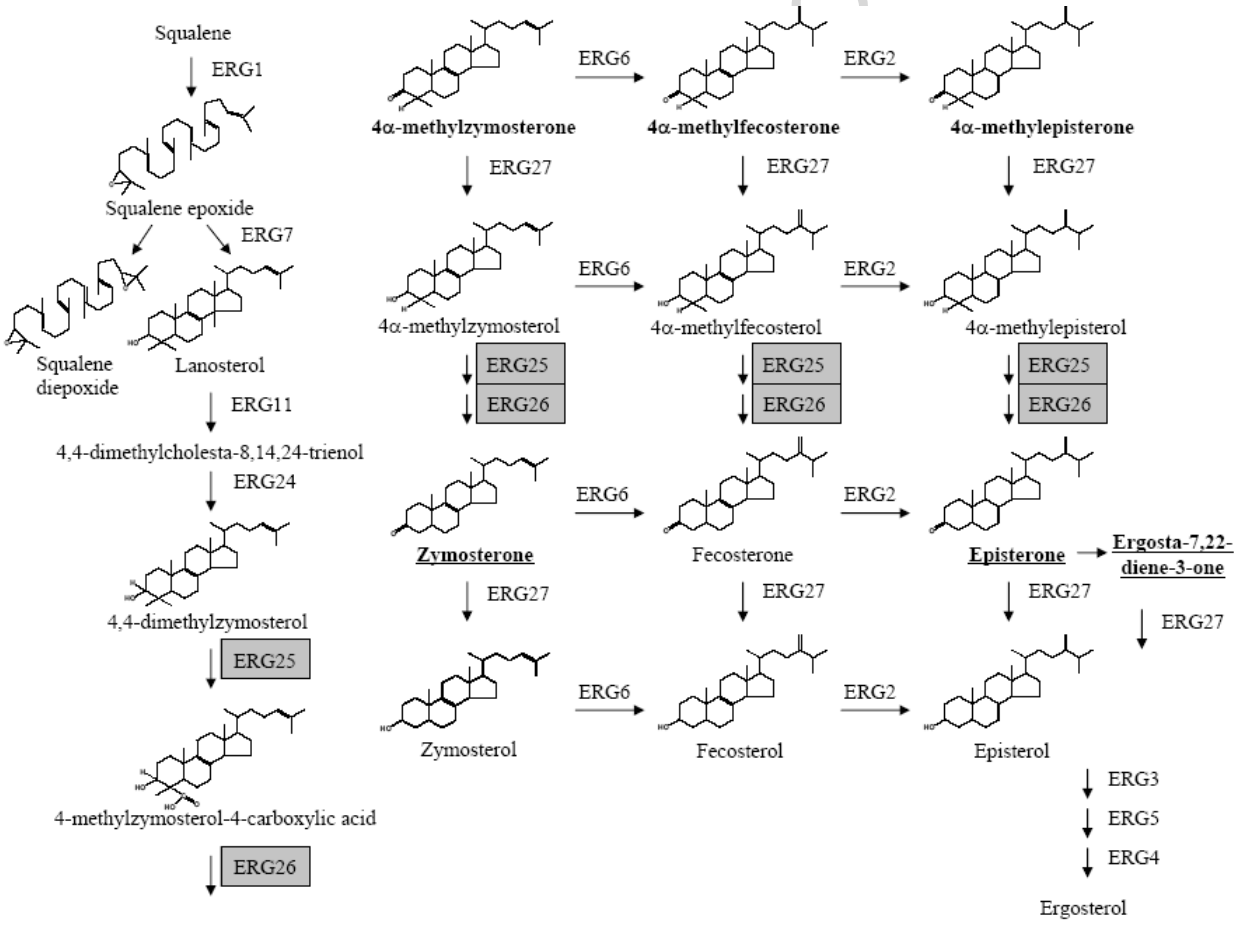
Table 6

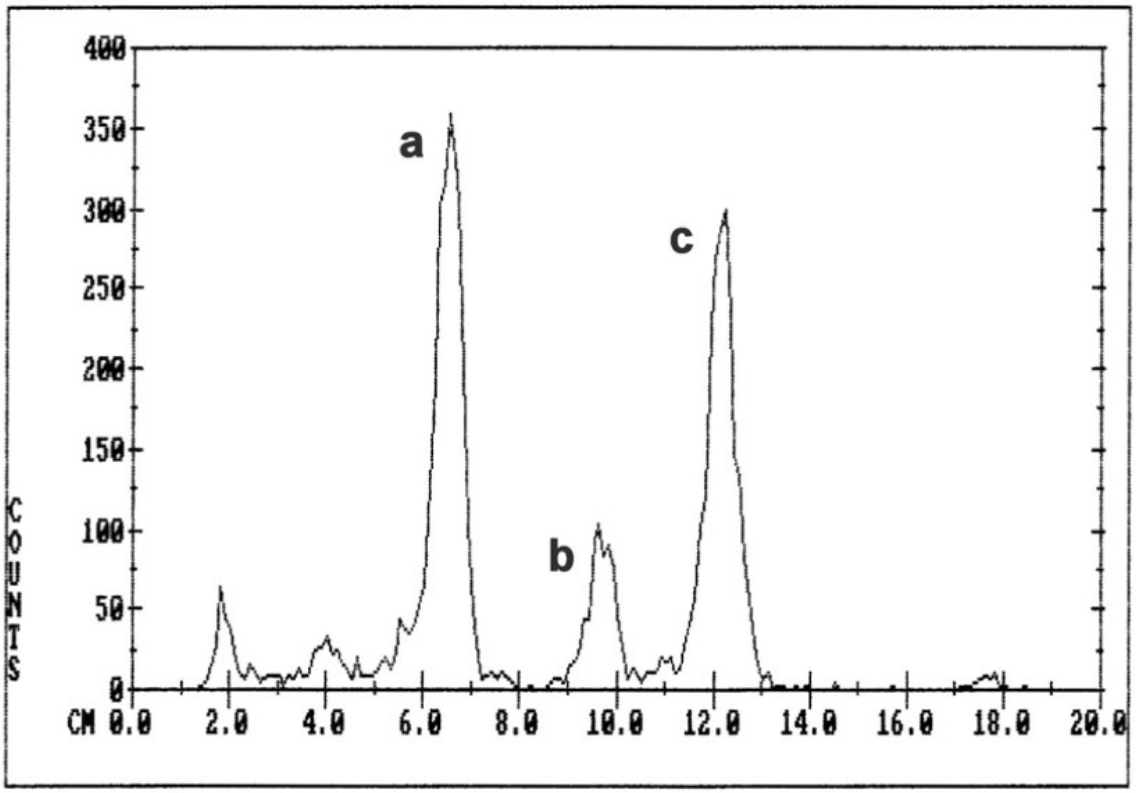
Incubation of homogenates with [^{14}C]3-ketosteroid 2

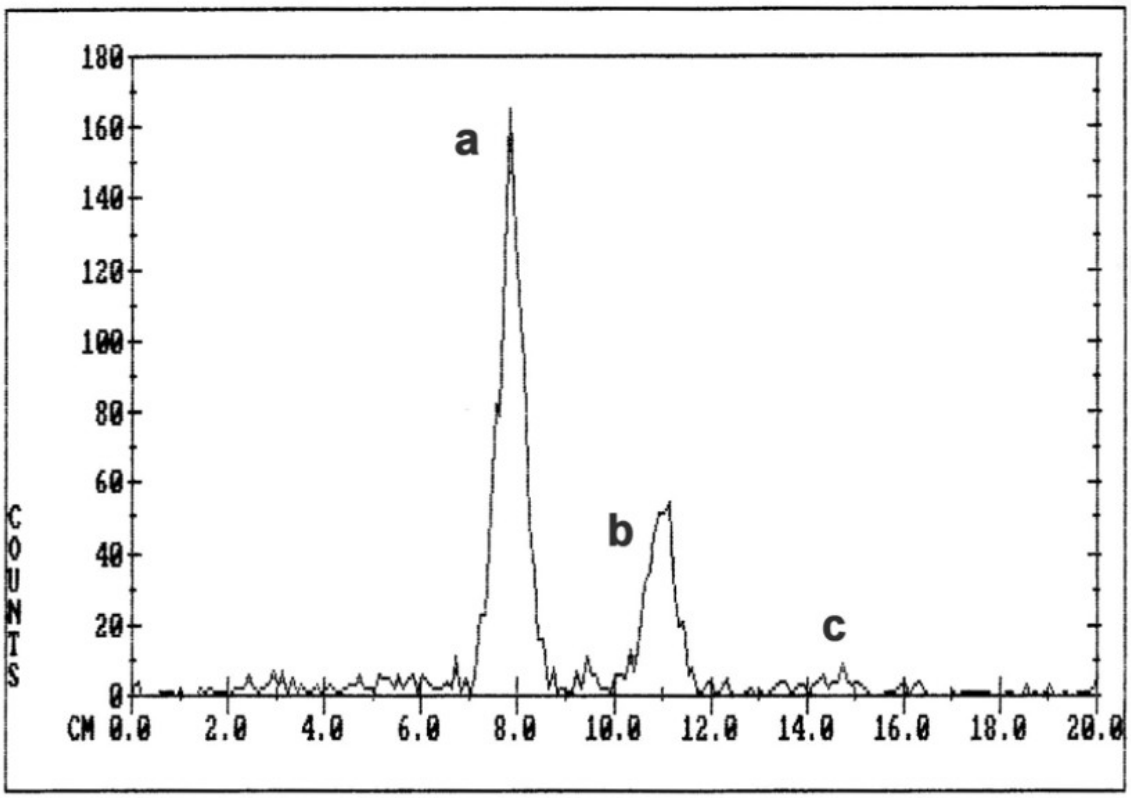
Strain	% radioactivity in nonsaponifiable lipids	
	3-ketosteroid 2	3-hydroxysteroid 2*
SCY876 (wt)	34.70	65.30
STY2 (*erg7*)	49.01	50.99
STY32 (*erg7*/pERG7)	46.74	53.26
FY1679-06C (wt)	32.57	67.43
GDY7 (*erg7*)	53.42	46.58
SMY8 (*erg7*)	30.01	69.99
SMY8 [pSM61.21] (*erg7*/p*ERG7*)	27.07	72.93

* 3-hydroxysteroid 2 was undistinguishable from ergosterol on TLC

Results are the means of at least two separated experiments with duplicate incubations, each. The maximum deviations from the mean were less then 10%.







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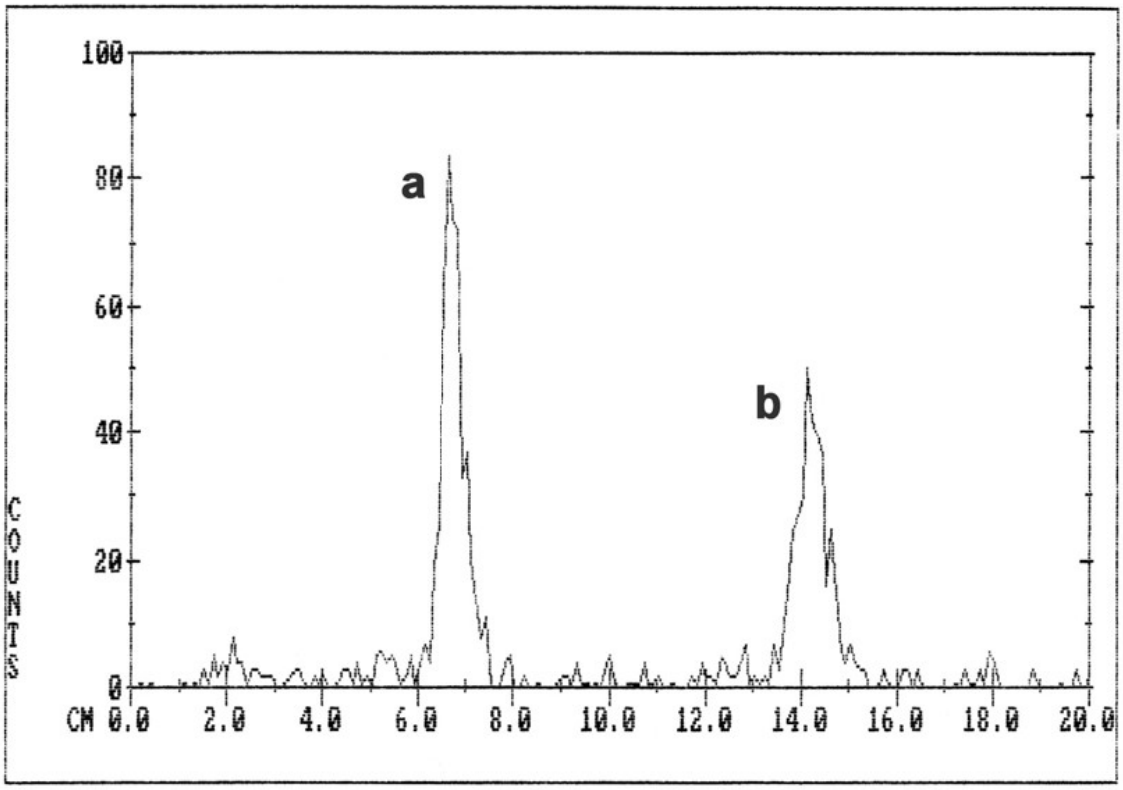

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