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The Regulation of Activity of Main Mevalonic Acid Pathway Enzymes: Farnesyl Diphosphate Synthase, 3-Hydroxy-3-methylglutaryl-CoA Reductase, and Squalene Synthase in Yeast *Saccharomyces cerevisiae*

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The co-regulation of the main mevalonic acid pathway enzymes was investigated in the yeast *Saccharomyces cerevisiae*. It was found that a 6-fold increase in FPPS activity compared with that of the wild-type strain FL100 did not cause significant changes in HMG-CoA reductase activity, while the amounts of synthesized dolichols and ergosterol increased by 80 and 32%, respectively. The disruption of the *SQS* gene in the strain grown in the presence of ergosterol repressed the activities of both FPP synthase and HMG-CoA reductase to a comparable degree, whereas in the same strain starved for ergosterol the activity of FPPS was 10-fold higher and HMG-CoA reductase activity was practically unchanged. We show that FPPS is the enzyme that regulates the flow rate of synthesized mevalonic acid pathway products independent of HMG-CoA reductase and SQS. © 2000 Academic Press

A precisely tuned mechanism regulates the biosynthesis of mevalonic acid pathway intermediates, the precursors of isoprenoid groups that are incorporated into more than a dozen classes of end products. Among them are sterols (especially ergosterol in yeast and cholesterol in animals) involved in membrane structure; haem A and ubiquinone, which participate in electron transport; dolichol, required for protein glycosylation; and isopentyladenine, present in some tRNAs and intracellular messengers such as cytokines in plants, farnesylated mating factors in yeast, juvenile hormones in insects, and steroid hormones in animals. The discovery that growth-regulating p^{21ras} proteins (1, 3), encoded by *ras* protooncogenes, oncogenes, and nuclear envelope proteins are covalently bound to farnesyl residues [4], which anchor them to cell membranes, additionally heightened the interest in the regulatory importance of the mevalonic acid pathway.

To ensure a constant production of the multiple isoprenoid compounds at all stages of growth, cells must precisely regulate the level of activity of enzymes to prevent accumulation of potentially toxic intermediates (5). In the past few years some genes that control synthesis of these intermediates have been cloned, and the molecular mechanisms for intermediate regulation are beginning to be known. Yeast and especially *Saccharomyces cerevisiae* are very suitable organisms for investigating the genes and gene products required in lipid metabolism. Their metabolic pathways are identical to those of higher organisms. Moreover, obtaining mutants with defects in defined enzymes or introducing additional copies of the enzyme of interest is a routine work.

In this study, we investigated how the overexpression of farnesyl diphosphate synthase (FPPS, mevalonic acid branch point enzyme) affects the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the other crucial enzyme, and how the disruption of the gene encoding squalene synthase (SQS, first committed enzyme in sterol synthesis) affects the activity of both HMG-CoA reductase and FPPS. Moreover, the changes in ergosterol and dolichol syntheses resulting from a disturbance in mevalonic acid pathway are discussed.

MATERIALS AND METHODS

Saccharomyces cerevisiae strains. For FL100 MAT a (ATCC 28383), FL100 [pCC2] plasmid pCC2 was a pUC19 derivative bearing the yeast 2 μm DNA and the *URA3* and *ERG20* genes (6). For *erg9::HIS3 ura3-1,his3-1,leu2-1,ade2-1,aux32,* the disruption of the squalene synthase gene resulted in an inability to perform ergosterol synthesis. The additional aux32 mutation in *erg9::HIS3* strain allows sterol uptake in aerobiosis (7).

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TABLE 1

Farnesyl Diphosphate Synthase and HMG-CoA Reductase Activities in the Wild-Type and Mutants of Yeast *Saccharomyces cerevisiae*

Yeast strain	FPPS activity[a] (nmol/mg/min)	HMG-CoA reductase activity[b] (nmol/mg/min)
FL100	1.21	1.13
FL100 [pCC2]	7.23	1.38
erg9::HIS3 + ergosterol	0.27	0.20
erg9::HIS3 − ergosterol	2.80	0.28

Note. Numbers are means of six[a] and four[b] independent experiments.

Media and growth conditions. Wild-type yeast strain was grown on YPG medium (1% bacto peptone, 1% yeast extract, and 2% glucose). FL100 [pCC2] was grown on 2% glucose and 0.67 g of yeast nitrogen base/100 ml medium without uracil supplemented with amino acids. *erg9::HIS3* was grown on YPG medium supplemented with ergosterol (2 mg/ml) in 1% Tween 80. For ergosterol starvation strain was harvested at a late log phase and transferred to YNB medium supplemented with amino acids, deprived of ergosterol and Tween 80, and cultivated for 24 h.

FPP synthase assay. Yeast cells were harvested at the exponential growth phase and washed with ice-cold 50 mM phosphate buffer, pH 7.5. Cells were disrupted with glass beads in the phosphate buffer containing 5 mM iodoacetamide. Cell debris were discarded by 12,000*g* centrifugation for 5 min at 4°C. The supernatant was again centrifuged at 12,000*g* for 30 min at 4°C and at 110,000*g* for 2 h at 4°C. The resulting supernatant was the enzyme source.

The enzyme assay was carried out according to Karst (8) with modifications. The incubation mixture in a final volume of 100 μl contained 50 mM phosphate buffer, pH 7.5, 1 mM $MgCl_2$, 5 mM iodoacetamide, 30 nmol of GPP, 6 nmol of IPP, 1×10^5 cpm of [^{14}C] IPP (sp act, 52 mCi/mmol), and 150 μg of crude enzyme. The incubation lasted for 5 min at 37°C. The sample was ice-chilled, then 0.5 ml of water was added, followed by 1 ml of hexane and 0.2 ml of 1 N HCl (to dephosphorylate products), and the mixture was shaken for 15 min at 37°C. The mixture was ice-chilled and vigorously mixed. The separated upper phase was washed three times with water and subjected to liquid scintillation counting.

HMG-CoA reductase activity assay. Yeast cells were grown on YPG medium to an optical density at 600 nm of 0.8 to 2. The pelleted cells were washed twice with ice-cold 50 mM Tris–HCl (pH 7.5). The cells were broken with glass beads by vigorous vortexing. The supernatant fraction was centrifuged at 2000*g* for 2 min at 4°C and served as enzyme source.

The activity of HMG-CoA reductase was estimated by measuring the conversion of [^{14}C]HMG-CoA to [^{14}C]mevalonate according to Thorsness (9). The incubation mixture in a final volume of 100 μl contained 50 mM Tris–HCl, pH 7.5, 5 mM dithiothreitol, 200 μM NADPH, 300 μM DL-[^{14}C]HMG-CoA (1 μCi/μmol), 20 mM glucose-6-phosphate, and 9.75 mU of glucose 6-phosphate dehydrogenase per milliliter for regeneration of NADPH. The reaction was started by addition of 400 μg of protein and was carried out for 5 min at 37°C. The reaction was stopped by addition of 20 μl of 6 N HCl and the mixture was further incubated for 15 min at 37°C to allow conversion of the reaction product, mevalonate, to mevalonolactone. The product and substrate were separated on a Bio-Rex 5 column. Mevalonolactone was eluted from the column with water washes. The amount of radiolabeled product was determined by scintillation counting.

Characterization and quantification of dolichols synthesized in vivo. The membrane fraction prepared as described (10) was extracted with chloroform:methanol 3:2 (v/v). Denatured protein was discarded by centrifugation. The organic supernatant was washed three times with 10 mM EDTA in 0.9% NaCl and evaporated to dryness. The extracted lipids were dissolved in hexane and applied to a silica gel column equilibrated with hexane. A step-wise gradient of 3, 8, 12, and 15% diethyl ether in hexane gave an almost pure fraction of dolichols subjected subsequently to HPLC analysis. HPLC was performed on ODS Hypersil 3 μm 60 × 4.5 mm column (Knauer) at a flow rate 1.5 ml/min in linear gradient program from A: methanol:isopropanol:water 12:8:1 (v/v/v) to 60% B: hexane:isopropanol 7:3 (v/v) in 30 min. The amount of dolichols was estimated against internal standard 0.1 mM Dol_{23}.

Determination of ergosterol level. The amount of sterols with 5–7 dienic system was determined spectrophotometrically. Yeast (1 g of wet weight) was hydrolyzed in 2 ml of 60% KOH in the presence of 5 ml of methanol at 70°C for 2 h. Then the mixture was vigorously shaken with 10 ml of hexane. After centrifugation the upper phase was saved and the lower again extracted with hexane. Combined hexane phases were evaporated to dryness and lipids suspended in 1 ml of ethanol. The molar extinction coefficient at 281.5 is 11,500 (11). With this technique ergosterol and ergosta-5-7-enol are measured but they represent about 70% of the total sterol amount in wild-type yeast.

RESULTS

The activity of HMG-CoA reductase was estimated in the wild-type yeast strain FL100 and the strain with overexpressed FPPS gene. Table 1 shows that at almost 6-fold increase in the FPPS activity reductase activity was changed by about 20%. Totally different results were obtained compared to the activities of both enzymes in the strain with disruption of squalene synthase gene cultivated in a medium supplemented with ergosterol. The losses of HMG-CoA reductase and FPP synthase activities were 82.2 and 77.7% (respectively). The most striking results, however, were obtained on measuring the activities of both enzymes in the same strain but starved for ergosterol. The loss of HMG-CoA reductase activity was practically unchanged (75.3%) while the activity of FPP synthase increased to 231.4% in comparison with the wild-type strain.

TABLE 2

Synthesis of Dolichols and Ergosterol Level in Wild-Type and Mutants of Yeast *Saccharomyces cerevisiae*

Yeast strain	Dolichol (μg/mg protein)	Ergosterol (μg/g)
FL100	0.236	177.48
FL100 [pCC2]	0.424	235.00
erg9::HIS3 + ergosterol	0.320	53.12
erg9::HIS3 − ergosterol	0.370	32.71

Note. Dolichols were extracted, purified, and quantified as described under Materials and Methods. The amount of ergosterol was determined from the absorbance at 281.5 nm.

AcetylCoA + Acetoacetyl CoA

↓

HMG - CoA

↓

Mevalonate

↓

Mevalonate-PP

↓

Isopentenyl-PP→ Isopenten l adenine (tRNA)

↓

Geranyl-PP

↓

Farnesyl-PP } dolichol, haem A, ubiquinone, franesylated proteins (Ras, laminA, laminB, mating factors in yeast, trimeric and monomeric GTP-binding proteins, certain kinases)

↓

Squalene

↓

ergosterol, cholesterol

FIG. 1. The mevalonic acid pathway.

Table 2 shows that the increased amount of FPP (in strain FL100 [pCC2]) may be transformed in ergosterol and incorporated into dolichols (about 32 and 80% increase compared to FL100, respectively). The observed increased dolichol synthesis (35.6% versus FL100) in the strain with disruption of *SQS* gene cultivated in the presence of ergosterol possibly results from the accumulation of FPP brought about by the block of FPP flux to the sterol pathway (Fig. 1). Even greater accumulation of dolichols (56.7%) has been observed in the same strain starved for ergosterol. Under these conditions the activity of FPP synthase was 10-fold higher.

The wild-type yeast strain has a typical dolichol family with dominating Dol_{16} (Fig. 2A), while dolichols synthesized by the strain with overexpression of the *ERG20* gene and with disruption of the squalene synthase gene present different patterns (Fig. 2B). Instead of one dominating dolichol, they have equal amounts of Dol_{15} and Dol_{16} (estimated by peak integration method; data not shown).

DISCUSSION

Investigations performed during the last three decades firmly established that the major regulatory enzyme of the mevalonic acid pathway is HMG-CoA reductase. However, in light of growing evidence, the involvement of reductase in the regulation of the biosynthesis of mevalonate-derived compounds is being

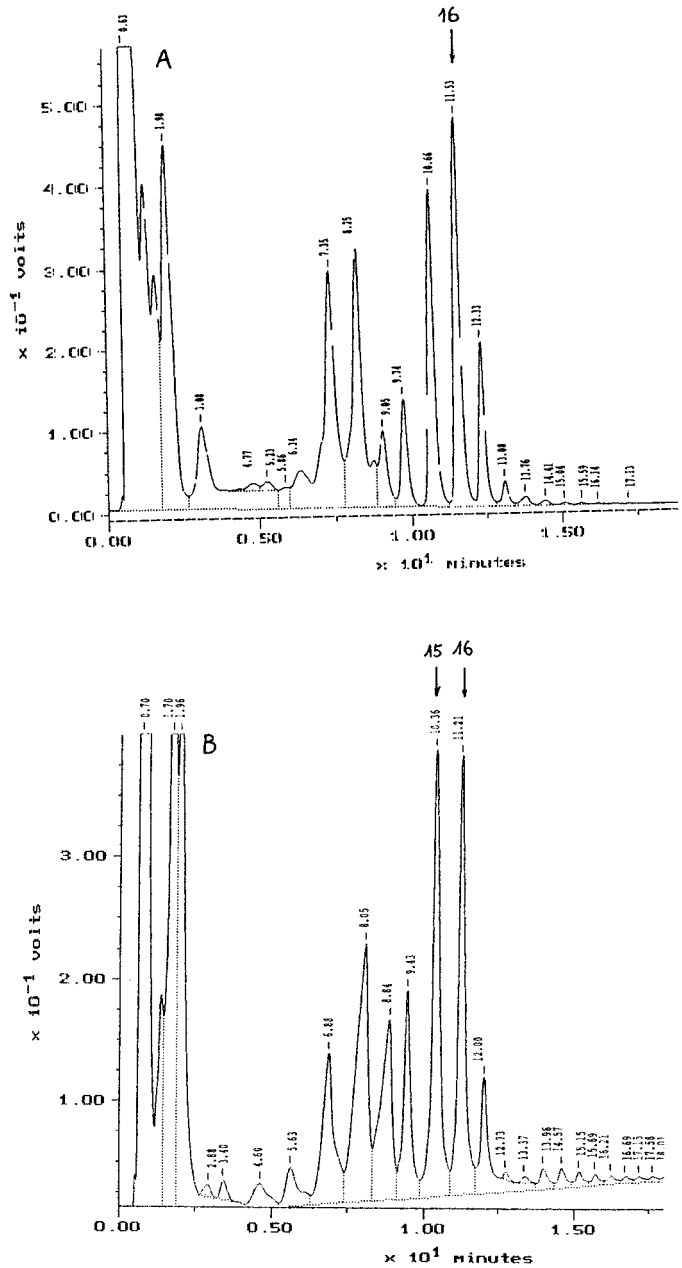

FIG. 2. HPLC analysis of dolichols synthesized by the wild-type and mutants of yeast *Saccharomyces cerevisiae.* (A) FL100; (B) FL100 [pCC2] and *erg9::HIS3.* Dolichols were extracted and purified as described under Materials and Methods. Numbers over the peaks represent retention times. Arrow with number indicates the amount of isoprene units of dolichol.

questioned. A number of experimental data show that biosynthesis of dolichols and ubiquinones, as well as isoprenylated proteins, is regulated by enzymes distal to HMG-CoA reductase. To obtain more information on the actual role of HMG-CoA reductase and the possibility of mevalonic acid pathway regulatory system manipulation (which could be useful in treating certain forms of human diseases, e.g., cancer or heart failure), the influence of two other candidates has been investigated: one—the pathway branch point enzyme farnesyl diphosphate synthase and the other squalene synthase—the first commited enzyme in sterol biosynthesis.

Yeast has two isozymes of HMG-CoA reductase called Hmg1p and Hmg2p encoded by the *HMG1* and *HMG2* genes, respectively (12). The Hmg1p isozyme is extremely stable while Hmg2p displays rapid, regulated degradation. In aerobic growth the proportion of Hmg1p in the cell is high and that of Hmg2p low.

Table 1 shows that the yeast strain bearing the *ERG20* gene on a multicopy plasmid has almost 6-fold higher FPPS activity than a control wild-type strain. Simultaneously the HMG-CoA reductase activity was changed only by about 20%. This is not a surprise since signals for Hmg1p feedback control regulation are derived from early mevalonate pathway products, before the production of squalene (in this case FPP). When oxygen is available there is efficient production of late (postsqualene) products of the pathway and consequently greater feedback inhibition of Hmg2p synthesis. So the net effect of about 20% change in the HMG-CoA reductase activity is understandable.

Such immense increase in FPPS activity correlated with a significant elevation in dolichol and ergosterol synthesis (about 80 and 32% higher, respectively). In a well-aerated yeast cell, all of the ergosterol that a cell needs is directly synthesized by the mevalonate pathway and furthermore, the cell can safely store any excess ergosterol as inert acyl esters (13). Moreover, the excess farnesyl groups are incorporated in dolichols (80% increase) so potentially cytotoxic products such as squalene and farnesol are neutralized. These results suggest that farnesyl diphosphate synthase, independent of HMG-CoA reductase, is responsible for directing of FPP as a substrate for squalene synthase and *cis*-prenyltransferase, acting as a rate-limiting factor in the synthesis of both groups of compounds. Nevertheless, Table 1 documents that the disruption of the squalene synthase gene (when the strain bearing disrupted *SQS* gene was cultivated in the presence of ergosterol) resulted in concurrently diminished activities of both FPP synthase and HMG-CoA reductase (78 and 83% repression, respectively). This strongly indicates that SQS is the enzyme determining the intermediate flow rates in the mevalonic acid pathway. When the early products of the pathway cannot be converted to ergosterol and its esters (wild-type strain FL100 synthesizes 177.48 μg/g of ergosterol while *erg9::HIS3* contains only 53.12 μg/g) and synthesis of dolichols is unable to absorb the bulk of FPP, both enzymes are repressed. An observed 80% increase in dolichol synthesis in strain FL100 [pCC2] could be possible since the HMG-CoA activity was not decreased (the early products of mevalonic acid pathway were synthesized so IPP synthesis should not be the limiting factor for *cis*-prenyltransferase). But the most surprising was finding that *erg9::HIS3* strain cultivated in the presence of ergosterol and then deprived of it reacted with over 10-fold increase in the farnesyl diphosphate synthase activity while the HMG-CoA reductase activity was increased only by 1.4-fold.

SREBPs (sterol regulatory element binding proteins) coordinately regulate transcription of HMG-CoA reductase as well as HMG-CoA synthase, farnesyl diphosphate synthase, and squalene synthase (14–16). However, current studies identified a distinct protein, termed "Red 25," overlapping the putative SRE-1 (sterol regulatory element 1) within the HMG-CoA reductase promoter which is believed to be involved in sterol-regulated transcription of reductase (17). Furthermore, a new sequence within the FPP synthase promoter, termed SRE-3, has been identified that is required for the induced levels of transcription observed in cells deprived of exogenous sterols. It has been found that SREBP binding to SRE-3 is stimulated in the presence of crude nuclear extract (16). Thus, the function of SREBP in different genes may be modulated by interaction with different transcription factors.

Results presented in the paper give evidence that earlier literature data indicating strictly coordinated regulation of the mevalonic acid pathway enzymes, i.e. HMG-CoA reductase, farnesyl diphosphate synthase, and squalene synthase with reductase considered to be the main regulatory enzyme of cholesterol (ergosterol in yeast) synthesis, do not find full confirmation. We show that FPP synthase, independent of HMG-CoA reductase and to a certain degree of squalene synthase, is the enzyme that responds to the greatest extent to the changing internal and external environmental conditions, adapting the yeast cell to them. The reason for this seems obvious if one considers the diversified cell functions in which its product—FPP—directly participates.

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