Characterization of Yeast Methyl Sterol Oxidase (ERG25) and Identification of a Human Homologue*

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A yeast mutant (LT06) was isolated that showed no growth on iron-limited medium but normal growth on iron-replete medium. A gene cloned from a genomic yeast library complemented the defect, allowing growth on low iron medium. Allelic segregation analysis demonstrated that the cloned gene was the normal allele rather than a high copy suppressor. A disruption mutant was nonviable, indicating that the gene was essential. Sequence analysis and functional assays indicated that the cloned gene was identical to ERG25, a gene that codes for methyl sterol oxidase. Incubation of LT06 in low iron medium resulted in marked changes in lipid metabolism, including the accumulation of fatty acids, triglycerides, methyl sterols, and other sterol precursors. A human homologue of ERG25 was cloned, sequenced, and mapped to human chromosome 4q32-34. Analysis of the data base with both ERG25 and the human homologue resulted in the identification of an extensive set of metal binding motifs with similarity to that seen in a family of membrane desaturases-hydroxylases. Western analysis using antibodies to an Erg25-GST fusion protein detected two proteins of 34 and 75 kDa. Both proteins are membrane bound and contain one N-glycosyl unit. Immunofluorescence data suggest that the proteins are present in the endoplasmic reticulum and plasma membrane. Although ERG25 transcripts are not iron regulated, there is a large increase in the concentration of transcript in the mutant LT06 grown in low iron medium. These results suggest that the enzyme is regulated not by iron but by an end product of the ergosterol pathway.

Over the past few years much progress has been made in elucidating the genetics and biochemistry of iron transport in the budding yeast Saccharomyces cerevisiae. Genes involved in high (1) and low affinity (2) iron transport have been cloned, their protein products have been identified, and their physiology has been examined. A molecule responsible for iron-depletion toxicity of streptonigrin. Surprisingly, a number of mutants obtained in that screen were capable of transporting iron at normal rates, indicating they had functional iron transporters and normal regulation of these genes. Because little is known regarding intracellular iron metabolism, we initiated study of these mutants in hopes of gaining insight into the enzymes and steps involved in cellular iron homeostasis. In this communication we report the isolation and properties of one of those genes. In the course of cloning the gene we discovered that it was identical to ERG25, a C-4 methyl sterol oxidase (5). We have cloned a human homologue of this gene and have defined a putative set of metal binding motifs. Antibodies were generated against an Erg25p-GST fusion protein and were used to localize the protein to two different subcellular organelles, plasma membrane and endoplasmic reticulum. Although the ERG25 transcript is not regulated by iron in wild type cells, there is an increase in the mutant LT06 transcript grown in low iron conditions.

MATERIALS AND METHODS

S. cerevisiae and Escherichia coli Strains—The yeast strains employed in this study were derived from DY150 and DY1457 as previously described (1). The cells were grown in YPD medium (1.0% yeast extract, 0.2% peptone, 2.0% glucose). In YPD made iron limited by the addition of bathophenanthroline sulfonate (BPS) (6), or in LIM medium, a synthetic medium that has defined concentrations of iron (6). Spheroplasts and a membrane preparation were obtained as described previously (7). Iron transport and ferri-reductase activity was assayed as described in Eide et al. (8).

DNA Sequencing—DNA was sequenced using the dideoxy sequencing method using Sequenase from U.S. Biochemical Corp. and the Applied Biosystems automated sequencer. The primers were universal M13 primers and primers prepared using previously obtained sequencing data. Both strands were sequenced.

Cloning and Recombinant Techniques—DNA transformations of E. coli and S. cerevisiae were performed by standard procedures (9). DNA fragments were isolated using Wizard mini-preps (Promega). The shuttle vector pTF63 was derived from Yeplac195 and contained the pBlue-script II polylinker (10). The Sau3A genomic library used in the complementation studies was described previously (1) and was the gift of Drs. D. Stillman and W. Ming. A SacI-HindIII fragment derived from the original complementing clone was subcloned into pTF63. The plasmid (pFET6) was cleaved separately with SacI and EagI and HindIII and Apal and then Exonuclease III or mung bean nuclease. After defined times the reaction was terminated, the ends of the plasmid were polished with T4 DNA polymerase, and the plasmid was religated. This

The abbreviations used are: GST, glutathione S-transferase; BPS, bathophenanthroline sulfonate; BPS(5), medium made low iron by the addition of BPS; PAGE, polyacrylamide gel electrophoresis.

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procedure allowed us to obtain a set of nested deletions. The subclones were tested for their ability to complement the low iron growth phenotype of LT06. The subclones were also used for sequencing.

A disruption mutant was generated by a one step χ-deletion of the ERG25 gene. A 410-base pair XbaI fragment was removed from the middle of the ERG25 open reading frame. The remaining portions of the gene were ligated to a 654-base pair of a plasmid vector, which contained TRP1 as a selectable marker. The plasmid was linearized with BshHI. The linearized fragment was used to transform DH5α diploid cells. The diploids were plated on trp− medium, and surviving colonies were examined for the gene disruption by Southern analysis. The diploids were sporulated, and the spores were dissected. The viability of the spores was examined on solid media.

Cloning of an ERG25 Human Homologue—Examination of the EST data base revealed an EST (EST03961, accession number T0672) isolated from a human fetal brain cDNA library that had high homology to pFET6. Based on the sequence of the EST, primers were designed (5′-GAAGATTTACACCAAC and 5′-TATGGAGATACCTGGCATA) that amplified a 196-base pair product. The conditions of amplification were: denaturation temperature, 94°C; annealing temperature, 55°C; elongation temperature, 72°C; for 20 ± 40 cycles using an Idaho Technology Thermal Cycler. The polymerase chain reaction product was gel purified, radiolabeled by random priming, and used to probe a human intestinal cDNA library made in Lambda MAX (Clontech). Over 140,000 plaques were screened, and four positives were obtained. The inserts were excised by addition of a helper phage as per the directions of the manufacturer.

Fluorescence In Situ Hybridization—An EcoRI fragment from the human cDNA, which contained most of the open reading frame, was used to probe a P1 genomic library. Hybridization was performed under high stringency (65°C, 0.1 × SSC), and a single positive clone was obtained. Purified DNA was digested with selected enzymes. The identity of the P1 was confirmed by Southern analysis using the EcoRI fragment of the cDNA as a probe.

The P1 was labeled with biotin-14-dATP using the BioNick labeling system (Life Technologies Inc.) as per the manufacturer’s instructions. The biotinylated probe was used to stain Colcemid treated lymphocytes using the protocol of Pinke et al. (11). The preparations were examined by fluorescence microscopy, and images were captured by a cooled charged coupled device camera.

RNA Isolation and Northern Blots—Total RNA was isolated from yeast grown for 7–12 h in a low iron medium, BPS(5), or in a high iron-containing medium, YPD. RNA was separated by agarose gel electrophoresis, blotted onto nitrocellulose paper, and then probed with either a 3.4-kilobase fragment from P1 (insert from P1 to an actin probe) randomly labeled with 32P. The blot was washed with 0.5 × SSC, 0.1% SDS at 65°C and then subjected to autoradiography at 70°C.

Lipid Extraction and Thin Layer Analysis—Cells grown in YPD were washed and then transferred to BPS(S) medium for specified times. The cells were harvested and lyophilized, and lipids were extracted using the Bligh and Dyer procedure (21) outlined by Pinke et al. (12). Lipids dissolved in hexane were separated by thin layer chromatography using silica gel plates (Whatman). The plates were developed in petroleum ether:diethyl ether:acetic acid (85:15:1, v/v/v). The plates were dried, and the lipids were visualized by exposure to iodine vapor. For radiolabeling experiments, 50-ml cultures grown in YPD were washed and incubated in BPS(S) for 5 h. [2-14C]acetate was added (58 mCi/mM, 0.4 μCi/μl, Amersham Life Sciences), and the incubation continued for another 5 h. The cells were extracted with dimethyl sulfoxide-hexane, and the extracted lipids were analyzed by thin layer chromatography, and the distribution of radioactive spots was visualized by autoradiography.

Preparation of Antibodies—An Erg25p-GST fusion protein was obtained by ligating a 1.4-kilobase fragment of P1 into a pGEX internal fragment from pFET6, into a pGEX vector. This fragment codes for the last 76 amino acids of Erg25p. The new plasmid pGEX-FET6 was transformed into E. coli. The fusion protein was induced by addition of isopropl-1-thio-β-D-galactopyranoside to the culture to a final concentration of 1 mM. After 2 h of incubation, the culture was harvested, and the cells were disrupted by sonication. The protein was isolated using a glutathione affinity column obtained from Pharmacia Biotech Inc. The eluted protein was digested with thrombin (50 units, 2 h, 23°C). The sample was applied to SDS-PAGE, and a band corresponding to the predicted molecular weight of 7,000 daltons was observed. The GST-fusion protein was isolated by binding to glutathione columns and the eluted protein when treated with thrombin showed the expected mass of 7,000 daltons on SDS-PAGE.

Sequencing of the first ten amino acids confirmed that this protein was identical to that of putative fusion peptide. Acrylamide gel samples containing this peptide were mixed with Freund’s complete adjuvant and injected into rabbits. Serum was obtained and IgG purified by applying rabbit serum to a protein G column (Pierce). The IgG was then affinity purified by incubating the antisera with nitrocellulose blots containing SDS-PAGE purified Erg25p peptide. Antibody bound to the blots were eluted with 50 μl glycine, pH 2.8. The pH of the sample was brought to neutral capture of the IgG. The wells were washed and the nitrocellulose was blocked overnight at 4°C by the addition of 5.0% nonfat dry milk dissolved in 20 mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20, pH 8.0. The blots were incubated with primary antibody (15 μg/mL C4) diluted in 4% milk, 0.1% Tween 20, 0.05% Sigma sodium azide and 5% nonfat dry milk in Tris-HCl, pH 8.0 (31) for 2 h at room temperature. The filters were washed and developed using the ECL procedure (Amersham Life Science) as per the manufacturer’s instructions. Samples were treated with endoglycosidase-H according to the manufacturer’s instructions (New England Biolabs).

Immunofluorescence—Cells were incubated in 3.7% formaldehyde for 2 h at room temperature. The cells were washed and spheroplasts were prepared as described above. The cells were washed and incubated in 1.2 M sorbitol, 40 mM potassium phosphate, 0.5 mM MgCl2, pH 6.5, and then allowed to attach to poly-l-lysine-treated cover slips for 10 min. The cover slips were incubated with 3% formaldehyde in phosphate-buffered saline for 20 min, washed, and then permeabilized by the addition of 0.05% saponin in phosphate-buffered saline containing 10 mM glycine and 0.1% bovine serum albumin. After 10 min the cover slips were washed and then incubated with affinity purified rabbit antibody. The cover slips were incubated with the primary antibody for 60 min, washed, and then incubated with a Texas Red-conjugated goat anti-rabbit antibody (Molecular Probes) for an additional 60 min. The cover slips were washed and sealed with nail polish, and cells were visualized using a Nikon inverted fluorescence microscope with a Zeiss 100 × oil immersion objective. Images (512 × 512) were acquired using a Photometrics cooled charge coupled device camera and a Macintosh workstation running Oncor image three-dimensional cytometry software. A multi-dye filter set used in which excitation filters of 575 nm (Texas Red) were selected from a computer-controlled filter wheel in conjunction with a multi-wavelength emitter and dichromatic filter set (640 nm, XF56 set from Omega Optical). Out-of-focus blur was removed by adjacent plane Fast Fourier deconvolution using the inverse of the modulation transfer function of the microscope. Adjacent planes were taken at 0.5-micron intervals under control of the Oncor imaging software package. Prior to deconvolution, all images were corrected for background and flat field. Images were then scaled to 256 levels of gray before output to a film recorder.

RESULTS

The original mutant, LT06-1, was isolated following streptonigrin enrichment and replica plating on low and high iron media. The mutant showed no growth on low iron medium (BPS(S)) and normal growth on YPD. Analysis of iron accumulation revealed, however, that the mutant had normal levels of both the high affinity iron transport system and ferrireductase activity (data not shown), suggesting that the low iron growth defect was not in iron transport but in some intracellular process.

Because the mutant was isolated using the chelator BPS to generate iron-limited medium, the possibility existed that lack of growth was not iron-specific. Although BPS is a high affinity iron chelator, it can also chelate other metals, particularly Zn2+, and to a lesser degree Cu2+. To determine if the mutant phenotype was iron-dependent, the mutant was plated on LIM medium, which could be made deficient in a number of different transition metals. When medium was made deficient in Zn2+, both mutant and wild type showed similar degrees in growth retardation (data not shown). This observation suggests that the defective growth on low iron medium is not due to a generic stress response.

The original mutant was backcrossed twice with wild type cells to assure that we were studying a single mutation. Diploids between the parental wild type and the mutant did not show the mutant phenotype, indicating that it is recessive.
When sporulated, the low iron phenotype segregated 2:2, indicating that the low iron growth phenotype was due to a single gene defect.

The backcrossed mutant (LT06-2) was transformed with a genomic library that contained the URA3 gene as a selectable marker. Colonies capable of growing in the absence of uracil were replica-plated onto BPS(S). Surviving colonies were picked and then grown in the presence of 5-fluoroorotic acid to determine if growth on low iron medium was plasmid-dependent: 37 colonies were found that required the plasmid for low iron growth. Plasmids isolated from 12 of these colonies were used to transform E. coli, and the plasmids rescued from E. coli were used to transform yeast. All of the isolated plasmids were able to confer low iron growth to the mutant. The isolated plasmids appeared similar by restriction analysis, and therefore only one of the plasmids was taken for further analysis.

The plasmid was subcloned by both restriction enzymes and exonuclease digestion to find the minimum size required for complementation of the low iron growth phenotype. The fragments were cloned into the vector pTF63, and the mutant LT06-2 transformed with the recombinant plasmid (pFET6) was examined for growth on high and low iron media. Digestion of the plasmid with XbaI or KpnI, which cut the plasmid in half, destroyed the ability to confer low iron growth. Exonuclease digestions localized the active fragment to a region of 1.4 kilobases (data not shown). This region was used for further genetic studies and for sequencing.

Transfection of the mutant with both high copy and integrated plasmids conferred the ability to grow on BPS(S), indicating that the isolated fragment was allelic to the original mutation and not a high copy suppressor. To confirm this view we performed allelic segregation. Using an integrative plasmid a URA3 gene was inserted next to the FET6 gene in the parental strain DY1457. We then crossed this new strain with LT06-2 and sporulated 30 diploids. The segregation of wild type and mutant phenotypes was 2:2. All of the wild type and none of the mutants were ura, indicating that the cloned gene was allelic to the mutant gene.

We attempted to create a disruption mutant by inserting a TRP gene next to the chromosomal copy of ERG25 using homologous recombination. Efforts to do so using haploids were unsuccessful, leading to the suggestion that a null may be lethal. To examine that possibility we transformed the insertion construct into diploids and selected for growth on trp medium. Colonies exhibiting trp prototrophy were examined for insertion of the TRP gene into ERG25 gene by Southern analysis. Those that showed the expected pattern were sporulated. None of the dissected haploids were capable of growing on trp medium even in high iron medium, whereas trp haploids were capable of growing on both low and high iron media. The diploid carrying the insertion deletion was then transformed with a ura FET6 plasmid. Diploids isolated on ura , trp medium were dissected, and haploids were sporulated. As expected all of the haploids could grow on low iron medium and were ura, and half of these could grow on trp medium. Each of the dissected haploids were grown with 5-fluoroorotic acid to promote loss of the plasmid. None of the colonies that survived growth in 5-fluoroorotic acid could grow in the absence of trp. The segregation of trp:ura was 2:2, demonstrating independent assortment. This result suggests that the ERG25 gene was required for growth even on high iron medium. An observation that further suggests this point is that the original mutation exhibited a temperature-sensitive growth defect on YPD (data not shown). Increasing the iron content of the medium could not overcome the growth defect at higher temperatures.

Sequence Analysis of FET6—The pFET6 was sequenced using the dideoxy procedure and DNA obtained from the exodigestions. The sequence revealed an open reading frame of 927 nucleotides. The deduced amino acid sequence suggests the protein has 309 amino acids, leading to a molecular weight of 36,455 (Fig. 1). Hydropathy analysis indicates that the protein may be a polytopic membrane protein with as many as four transmembrane regions (Fig. 1B). The amino terminus, however, does not appear to contain a leader sequence, and the protein has one potential N-glycosylation site. This sequence was found to be identical to that of ERG25, a methyl sterol oxidase isolated by Bard et al. (5). Throughout this report this gene will be referred to as H-ERG25, and the human homologue will be referred to as H-ERG25. In B is a hydrophathy analysis of the deduced amino acid sequence performed using the algorithm of Doolittle and Kyte (23). The positions of the histidine clusters is denoted by the horizontal bars.

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Examination of the data base revealed homology of the translated ERG25 sequence to an anonymous mammalian EST. Using the sequence of the EST we generated a polymerase chain reaction product and used this product to screen a human intestinal cDNA library and isolate hybridizing clones. The inserts from the clones were sequenced, and the resulting sequence was compared with the yeast sequence. The two sequences show an identity of 38% and a similarity of 62%.
cDNA was used to isolate a P1 clone, and that clone was used in fluorescence in situ hybridization analysis (data not shown). This analysis indicated that the putative human homologue of ERG25 mapped to chromosome 4q32-34. The deduced human protein also has at least four transmembrane loops, lacks a demonstrable leader sequence, and has two potential N-glycosylation sites. Of particular interest is that the human and yeast proteins shows identity in the four marked histidine clusters and in the hydrophathy profile.

Using both the human and yeast sequence, we examined the data base for homologous proteins or motifs. Seven proteins were identified that showed significant similarity to the deduced human and yeast proteins. Three of the proteins are closely related C-5 sterol desaturases, ERG3 from S. cerevisiae, Candida glabrata, and Arabadopsis and SUR2, a gene that confers sensitivity to the ergosterol binding antibiotic syringomycin. Additionally three open reading frames from the Caenorhabditis elegans also showed homology, particularly in the spacing and composition of the histidine motifs (Fig. 2). The homology also extends outside the histidine regions. Four distinct histidine cluster motifs can be assigned to all the proteins that show homology to ERG3-ERG25 family. Typically histidine motifs are associated with metal binding. Recently Shanklin and colleagues defined a similar histidine cluster motif present in an extended family of membrane fatty acid desaturases (15). These motifs exhibit a defined topology: the first two histidine motifs are on one cytoplasmic loop, whereas the last histidine motif was found on a large cytoplasmic COOH-terminal loop. These motifs are found in different eucaryotes, fungi, mammals, and ticks but have not been identified in plants. The authors presented data indicating that the histidine motifs bind oxo-diiron (Fe-O-Fe) (16). The membrane desaturase motifs were not recognized using the Blast and Motifs programs and the ERG3-ERG25 sequences to search the data base. The spacing of amino acids within the histidine motifs and the homology of amino acids outside of the motifs are different (Fig. 2B). Of interest is that where the desaturase family has three potential metal binding motifs, the ERG3-ERG25 family shows four motifs. Motif III is particularly novel; the ERG3 members and two of the worm homologues have His as a putative metal coordinating ligand, whereas ERG25, the human homologue, and one of the worms have aspartic acid, the carboxyl anion being a potential metal binding ligand. SUR2, a gene whose function is unknown (17) has an alanine in this position, so the metal binding status of this domain is unclear. The distribution of these motifs is similar to those seen in the desaturase family: motifs I and II and motifs III and IV are found on different cytosolic loops.

Sterol and Lipid Distribution in Mutant LT06-2 Incubated in Low Iron Medium—We examined the distribution of lipids in LT06-2 grown in YPD medium and then transferred to BPS(5). At selected times after the transfer to low iron medium, lipids were extracted and nonsaponified lipids were examined by thin layer chromatography. Based on the position of standards, it appears that both triglycerides and free fatty acids accumulate in mutant cells (Fig. 3). Saponification reduces the level of triglycerides and increase the amounts of free fatty acids (data not shown). Analysis by gas chromatography mass spectrometry indicates that the ratio of palmitoleate to oleate is similar in the mutant and in the wild type (data not shown). Decreased levels of ergosterol and ergosterol-esters were observed as well as increased amounts of methylated sterols and squalene. Overexpression of Erg25p resulting from transformation with a high copy plasmid (pFET6) resulted in a pattern of lipids and sterols similar to that seen in wild type cells. We also examined de novo lipid synthesis in the mutant

Fig. 2. Potential metal binding motifs of the ERG3-ERG25 family and its similarity to the metal binding domains in the desaturase/hydroxylase family. In A is a comparison of the metal binding motifs of the ERG3-ERG25 family of proteins. The C. elegans sequences are the result of the C. elegans sequencing project. The accession numbers are Z66520, clone F 49E12.9 (WERM1), Z66520, clone F 49E12.10 (WERM1), and U40941, clone F 35C8.5 (WERM3). Worm 1 and 2 map to chromosome XII and are physically adjacent. They are probably the result of gene duplications. Worm 3 maps to chromosome X. The sequences were analyzed by a combination of the Blast, Motifs, and Prosite programs. In B is a comparison of the motifs in the ERG3-ERG25 family with those in the desaturase/hydroxylase family. The data for this motifs were obtained from Shanklin et al. (15). See text for further details. For the desaturases-hydroxylases, domain 1A is separated from 1B by 20–50 residues but is on the same cytosolic loop, and domain II is separated from 1A by 100–200 residues and is on a different cytosolic loop. For the ERG3-ERG25 family, domains I and II are on the same cytosolic loop separated by 9–14 residues, domains III and IV are on the same cytosolic loop separated by 9–16 residues, and domains I and II are separated from III and IV by 62–68 residues.

LT06-2 grown on low iron medium. Cells growing exponentially on YPD were transferred to low iron medium. After 5 h of incubation, the cells were then incubated for a further 5 h with [14C]acetate. The cells were harvested, and lipids were extracted and analyzed by thin layer chromatography. The data indicate little evidence for the appearance of ergosterol, whereas there is an accumulation of lanosterol and material that is under-represented in wild type cells (Fig. 4) that migrates in the same position as methylated sterols. As this study was being performed, Bard and colleagues isolated the gene for cholesterol methyl oxidase (5) and using a disruption mutant of ERG25 have identified the methylated sterol as 4′,4′-dimethyl zymosterol.

Analysis of ERG25 Transcripts—Northern analysis of yeast mRNA using an ERG25 probe indicates a transcript of 1.2 kilobases. In wild type cells the concentration of this transcript was unaffected by growth in iron-replete or iron-limited me-
Human fibroblast mRNA was examined using a probe made from the human cDNA. A single transcript of 2.0 kilobases was identified whose concentration in human skin fibroblasts was unaffected by growth in iron-limited or iron-replete medium (data not shown). Probing a human tissue blot we have found transcripts in all cell types and organs assayed. This result suggests that the transcript is ubiquitous.

The amount of ERG25 transcript in wild type yeast cells is unaffected by cellular iron concentration even when incubated in iron-free medium (BPS(0)). The mutant LT06-2, however, showed an increase in transcript when incubated in low iron medium. Densitometric analysis, normalizing for the amount of actin transcript, reveals a 10-fold increase in ERG25 transcripts in mutant cells grown in low iron medium. This observation suggests that the concentration of transcript is regulated not by iron but by either the accumulation of precursor or the deficit of product.

Cellular Localization of the Erg25 Protein—To characterize the Erg25p, antibodies were prepared against a bacterial expressed fusion protein and affinity purified. When yeast membrane and cytosolic fractions were probed with the antisera, no bands were detected in the cytosol, and two bands were detected in the membrane fraction. The result confirms the sequence data, which suggested that Erg25p is a membrane protein. We were, however, surprised to find that the antisera detected two bands, one of 36 kDa, slightly higher than predicted from the deduced sequence, and one of 75 kDa (Fig. 6). To rule out the possibility that the antibodies were directed at two different proteins, we eluted antibodies from both the high and low molecular weight bands and used those antibodies to probe new Westerns. Both eluted antibodies detected the same two bands, indicating that the antiserum is recognizing identical epitopes on both molecular weight forms. Treatment of membrane extracts with endoglycosidase-H resulted in a reduction in size for both the lower and upper bands. The magnitude of the reduction suggests the presence of one N-glycosyl unit, consistent with the prediction from the sequence analysis.

Fluorescence microscopy (Fig. 7) using affinity purified an-
tibodies indicates that Erg25p has both a perinuclear and peripheral distribution. Based on this analysis it appears that the Erg25p is associated with both the endoplasmic reticulum and the cell surface.

**DISCUSSION**

Using a selection system to isolate mutants defective in iron accumulation, we observed that the majority of mutants had no measurable defect in iron transport. This suggests that iron-limited growth results from defects in genes that code for iron-containing proteins or proteins involved in iron metabolism. Using complementation of low iron growth as a phenotype, we chose one specific mutant LT06 and cloned a gene that restored growth. Analysis of the cloned yeast gene and a human homologue suggested that these genes were members of a family of iron binding proteins. As this work was being done it was reported that the gene we had cloned was identical to that of ERG25, which has been putatively identified as a methyl sterol oxidase (5). Analysis of cellular lipids and sterols in the LT06 grown in low iron medium confirms that identification.

**We also observed increases in free fatty acids and triglycerides.** We think that these reflect the decrease in ergosterol synthesis and the hydrolysis of ergosterol-fatty acid esters. The accumulation of triglycerides may be a response to the increase in free fatty acids. It should be stated that identification of the ERG25 gene product as a methyl sterol oxidase has been made based on the accumulation of precursor sterols. It may well be that the ERG25 gene product is a subunit of a multifunctional enzyme complex. The exact biochemical step affected by this gene product will require in vitro biochemical analysis.

The fact that the original mutation showed a low iron phenotype suggests that the ERG25 gene product, a methyl sterol oxidase, is an iron-requiring enzyme. Analysis of the protein sequence reveals several motifs similar to those found in the desaturase/hydroxylase family. Not only are the motifs similar, but the arrangement of the motifs with respect to the transmembrane domains are similar. These motifs have been demonstrated to bind oxo-diiron (Fe-O-Fe), which is involved in electron transfer reactions with O₂ as the final acceptor. The desaturase reaction is an oxygen requiring reaction as is the C-5 sterol desaturase reaction and the methyl sterol oxidase step (for review see Ref. 18). The iron requirement for growth of the mutant, the similarity in potential metal binding domains, and the absolute requirement for oxygen suggest that the methyl oxidase and the C-5 sterol desaturase may also be oxo-diiron containing proteins. Of interest is that the mutation that results in a low iron phenotype does not occur within the motifs but rather in a putative transmembrane domain. The change from a proline to a leucine is, however, relatively close to motif 1, and it may be expected to affect the tertiary structure of the metal binding motif and thus its ability to ligand metals.

As might be expected for a metabolic pathway that is ubiquitous and essential, transcripts for the yeast enzyme and the putative mammalian homologue are ubiquitous and do not appear to be regulated by iron. This observation indicates that iron plays a catalytic rather than a regulatory role. What is surprising is that when the mutant is grown on low iron, there is an increase in transcript, whereas in the mutant grown on normal or high iron transcript is present at wild type levels. Because the transcript is not regulated by iron, this result
indicates that the transcript is increased as a result of the metabolic block. A reasonable prediction is that a decreased product of a subsequent step in the pathway is involved in regulating transcript level. Older studies in mammalian cells have shown that methyl sterol oxidase activity was affected by treatments that affected cholesterol metabolism (feeding of cholestyramine) (19). This regulation was suggested to be post-translational and dependent on a cytosolic protein, although the biochemical basis was not defined (20). In yeast ERG11, the gene responsible for lanosterol 14-demethylase activity, an immediate precursor, is regulated by a complex set of environmental stimuli, including carbon source, oxygen, and heme (21). It may be that ERG25 is similarly regulated. The presence of mutants in the later and early steps in the ergosterol pathway will be invaluable in elucidating the exact step in the pathway responsible for regulation.

The deduced amino acid sequence indicates that the Erg25p is a membrane protein. Generation of an antibody has allowed for that possibility to be tested. Western analysis indicates that all immunodetectable material is membrane-associated. The sequence indicates one potential NH2-terminal glycosylation site. Cleavage with endoglycosidase-H generates a smaller form that has a molecular weight identical to that of the deduced protein. This provides further evidence that this is an integral membrane protein. What was unexpected was to find two molecular mass forms of the protein, a 37-kDa moiety and a 75-kDa moiety. The high molecular mass form also shows a size reduction upon endoglycosidase-H treatment, but the size is only reduced by a few kilodaltons. Solubilization of membranes at temperatures ranging from 25 to 100 °C did not affect the size of the band, suggesting that it is not simply an aggregate of the 37-kDa protein (data not shown). The high molecular weight form can be found in both wild type cells and in cells that contain a high copy plasmid and overexpress the protein. Immunofluorescence of cells containing high copy plasmid shows fluorescence associated with both the endoplasmic reticulum and the cell surface. Association of Erg25p with the endoplasmic reticulum is expected, much of sterol metabolism is localized to that organelle, and inspection of the amino acid sequence reveals the presence of a putative endoplasmic reticulum retention sequence (KKTN) (22). We think, however, that the higher molecular weight form may be associated with the plasma membrane. If this hypothesis is correct then the increase in molecular weight may be the result of O-linked glycosylation, because removal of N-linked sugars had only a modest effect. It is not clear whether the higher molecular weight form has functional significance.

We initiated this study to examine the basis for the lack of growth of cells on low iron medium. We assumed that mutants not defective in iron transport would be in proteins that either require iron or were involved in iron metabolism. The first studied mutant, LT06, appears to fall in the latter category; the data are consistent with the hypothesis that it codes for a protein that requires iron for catalytic activity. The fact that such conditional mutants exist suggests that the concentration of intracellular free iron can vary greatly. These extreme variations can occur in the face of no obvious change in growth rate. Clearly the variation in intracellular iron content affects intracellular processes; the induction of the high affinity iron transport system being the most obvious. That the extremes in intracellular iron content would allow for the isolation of conditional mutants has not been appreciated. Based on these results the addition of selective media in conjunction with the variation in intracellular iron content should allow for the targeted isolation of specific classes of iron genes.

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