rapid communication

A novel gene conserved from yeast to humans is involved in sterol biosynthesis

D. Gachotte,* J. Eckstein,† R. Barbuch,† T. Hughes,§ C. Roberts,§ and M. Bard[1,*]

Department of Biology,* Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202; Department of Drug Disposition,† Eli Lilly and Co., Lilly Corporate Center, Indianapolis, IN 46285; and Rosetta Inpharmatics, Inc.,§ Kirkland, WA 98034

Abstract **The *ERG28* gene was originally identified by microarray expression profiling as possibly involved in the *Saccharomyces cerevisiae* sterol pathway. Microarray analyses suggested that the transcription pattern of *ERG28* closely followed that of genes involved in sterol synthesis. *ERG28* was also found in *Schizosaccharomyces pombe* and *Arabidopsis* as well as humans, and in the latter was shown to be highly expressed in adult testis tissue. All four proteins contain potential transmembrane domain(s). Gas chromatography-mass spectrometry analysis of an *ERG28*-deleted *S. cerevisiae* strain (which is slow growing but not auxotrophic for ergosterol) indicates a lesion in sterol C-4 demethylation. Sterol profiles indicate accumulation of 3-keto and carboxylic acid sterol intermediates, which are involved in removing the two C-4 methyl groups from the sterol A ring. Similar intermediates have previously been demonstrated to accumulate in *erg26* (sterol dehydrogenase/decarboxylase) and *erg27* (3-ketoreductase) mutants in yeast. We speculate that the role of the Erg28 protein (Erg28p) may be either to tether Erg26p and Erg27p to the endoplasmic reticulum or to facilitate interaction between these proteins.**—Gachotte, D., J. Eckstein, R. Barbuch, T. Hughes, C. Roberts, and M. Bard. **A novel gene conserved from yeast to humans is involved in sterol biosynthesis. *J. Lipid Res.* 2001.** 42: **150–154.**

Supplementary key words ergosterol • C-4 demethylation • *Saccharomyces cerevisiae*

All genes encoding known enzymes in the *Saccharomyces cerevisiae* ergosterol (ERG) biosynthetic pathway have now been cloned. Beginning with *ERG10*, which encodes acetoacetyl-CoA thiolase, and ending with *ERG4*, which encodes the sterol C-24 reductase, there are 22 genes responsible for interconversions leading to ERG (1). DNA transcriptional microarray analyses, however, showed that an uncharacterized *S. cerevisiae* open reading frame (ORF), *YER044c*, responded similarly to ERG biosynthetic genes when ERG biosynthesis was inhibited by mutation of ERG pathway genes or the addition of azoles (2). Deletion of the *YER044c* gene, now designated *ERG28*, resulted in induction of other genes in the ERG pathway consistent with a sterol defect. While *ERG28* is not essential for viability, it is required for a normal growth rate (3).

In yeast, plants, and animals, conversion of lanosterol to zymosterol requires demethylation of the C-14 carbon, and two rounds of demethylation at C-4 (4). The first step in removal of a single C-4 methyl group requires a C-4 sterol methyl oxidase, which converts each methyl group by a series of three oxidations to a carboxylic acid. A dehydrogenation at the C-3 hydroxy leads to the spontaneous loss of the C-4 carboxyl group as CO_2 catalyzed by the C-3 sterol dehydrogenase/C-4 decarboxylase. This decarboxylation results in a C-3 ketone that is converted back to the alcohol by the 3-ketoreductase. The genes encoding these enzymes have been cloned in our laboratory and are designated *ERG25*, *ERG26*, and *ERG27*, respectively (5–7). All three are essential because deletions in any one leads to sterol auxotrophy. In the present study, we demonstrate by gas chromatography-mass spectrometry (GC-MS) that the *ERG28* gene product is necessary for efficient C-4 demethylation of sterols. While significant amounts of ERG and ERG precursors accumulate in a deleted *erg28* strain, a number of novel intermediates occur which, using GC-MS, we identify as 3-keto and carboxylic acid sterol (CAS) intermediates, some of which have been observed in strains containing lesions in *ERG26* and *ERG27*, respectively. We suggest that the *ERG28* gene product may anchor the C-3 sterol dehydrogenase/C-4 decarboxylase and 3-ketoreductase enzymes to the endoplasmic reticulum (ER), because unlike most ERG biosynthetic enzymes these lack an obvious transmembrane domain.

Abbreviations: CAS, carboxylic acid sterols; ER, endoplasmic reticulum; ERG, ergosterol; GC-MS, gas chromatography-mass spectrometry; h*ERG28*, human *ERG28* gene; *sERG28*, *Saccharomyces ERG28* gene; TM, transmembrane; WT, wild type.

[1] To whom correspondence should be addressed.
e-mail: mbard@iupui.edu

MATERIALS AND METHODS

Construction of *erg28* deletion strains

Wild-type (WT) strains BY4741 and BY4742 have been previously described and are the parental strains used by the yeast deletion consortium to assess the essentiality of each yeast gene (8). R711 and R712 were isolated as *erg28* G418-resistant segregants of BY4743 heterozygous for *ERG28* (*Mat***a**/α *erg28*::*kanMX4/ERG28 his3*Δ*1his3*Δ*1 leu2*Δ*0/leu2*Δ*0 met15*Δ*0/MET15 lys2*Δ*0/LYS2 ura3*Δ*0/ura3*Δ*0*). Deletions were made according to the deletion module polymerase chain reaction (PCR) strategy (9), in which *ERG28*-specific primers 5′ and 3′ to the ORF, respectively, GATGTCCACGAGGTCTCTACATGAGGAGCAGTTTGCATCGTA CGCTGCAGGTCGAC and CGGTGTCGGTCTCGTAGAATCCAG TCGGAATCATGGCATCGATGAATTCGAGCTCG, were used to amplify a kanMX4 cassette such that the entire *ERG28* ORF was deleted and replaced by the kanamycin resistance (Kan^R) cassette. Verification of the deletion involved 5′ and 3′ flanking primers A (GCTACTATTTTTGACGTAAACGCAT) and D (TTA AATAATGTACGGAAGGTTTGGA) and the two primers common to the kanMX4 module, kanB (CTGGACCGAGGAGCC GTAAT) and kanC (TGATTTTGATGACGAGCGTAAT). Two other primers internal to the *ERG28* gene, primers B (TTCAAG TACATAGCCCCATAGAATC) and C (CATGTCTTATATGGTTGC CCTATTC), failed to generate PCR product in conjunction with the A and D primers, respectively, in the disrupted strain.

PCR amplification of the WT yeast Saccharomyces *ERG28* and human *ERG28* genes

A 1.3-kb *Eco*RI-*Bam*HI fragment containing the entire *Saccharomyces ERG28* (*sERG28*) ORF, 440 base pairs (bp) of promoter, and 319 bp of downstream DNA sequence was amplified by PCR and inserted into vector pRS306 (10) to give pIU1600. PCR was performed on a Perkin-Elmer (Norwalk, CT) 2400 thermocycler using *Pfu* DNA polymerase and the *sERG28* gene was sequenced by the Biochemistry Biotechnology Facility at the Indiana University School of Medicine (Indianapolis, IN), using a Perkin-Elmer Biosystems (Foster City, CA) model ABI 373 automated DNA sequencer.

Plasmid pDW394 containing the YEplac 195 vector backbone (11) into which the *HOR7* promoter and the *PGKt* terminator region were inserted was obtained from Acacia Biosciences (Richmond, CA). A 71-bp oligomer primer containing 50 bases of *HOR7* promoter and 21 bases of human *ERG28* (h*ERG28*) leader sequence just upstream of the ATG (TATCAAATCATACAGATA TTGTCAAAAAAAAAAAAGACTAATAATAAAAACACGTTTGAG GGGAGTCATGA) and a 79-bp oligomer containing 56 bases of the *PGKt* terminator region and 20 bases of the reverse complement of the h*ERG28* ORF termination region (TAAAGGATG GGGAAAGAGAAAAGAAAAAAATTGATCTATCGATTTCAATTT TAAAATTAAAGAGGAGAGACGACGAAGG) were used to PCR amplify human testis cDNA (InVitrogen, Carlsbad, CA) using a *Taq/Pfu* mixture. A yeast *erg28* null mutant was then cotransformed with the 570-bp PCR product and the pDW394 plasmid was gapped by *Hin*dIII and *Sph*I. Selection for *URA3* transformants results in the generation of circular recombinant plasmid after homologous recombination (12, 13). The resulting plasmid pDW394::hERG28 was sequenced with the *HOR7* (GCTCAC TATGTGACAGTTC) and *PGKt* primers (GGCATTAAAAGAGGA GCGAA) to confirm the h*ERG28* sequence. Inserts were confirmed by sequencing, using the ABI 377 automated DNA sequencer.

Strains, media, and transformations

Yeast strains were grown at 30°C in YPAD medium [1% yeast extract, 2% Bacto-peptone, 2% glucose, and adenine (20 mg/l)] and grown for approximately 18–20 h to an OD_{600} of 4–6. Transformants of *erg28* were grown on complete synthetic medium-uracil medium containing 0.67% yeast nitrogen base, 2% glucose, and amino acids and nitrogen base supplements at 0.8% (Bio 101, La Jolla, CA). Yeast strains were transformed by standard methods (14). The *Escherichia coli* strain DH5α (F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 phoA hsdR17*(r_k^-, m_k^+) *supE44*λ$^-$ *thi-1 gyrA96 relA1*) was transformed as previously described (14) and grown in Luria-Bertani medium with ampicillin (50 μg/ml) at 37°C.

Sterol extraction and analyses

Total lipids were extracted from cells grown overnight in the presence of acid-washed glass beads according to the method of Bligh and Dyer (15). Phospholipids were precipitated at −20°C in the presence of acetone–chloroform 10:1 (v/v) followed by centrifugation at 12,300 *g* as described previously (6). CAS present in the sterol extract were methylated with diazomethane generated from *N*-methyl-nitrosourea in a 40% KOH solution and extracted with ethyl ether. Free sterols, 3-keto sterols, CAS, and sterol esters were resolved on 60 TLC F_{254}-precoated silica gel plates (E. Merck, Darmstadt, Germany) with methylene chloride as the solvent system. Organic compounds were detected after spraying with a 0.1% berberin sulfate ethanolic solution after exposure to short wavelength ultraviolet light irradiation. Hydroxysterols, 3-keto sterols, and CAS fractions were eluted from silica plates using methylene chloride. Alternatively, for complementation analyses sterols were extracted by saponification of whole cells as described by Molzahn and Woods (16). This protocol was used to detect free and 3-keto sterols. GC-MS analyses of sterols were done with a Varian (Palo Alto, CA) 3400 gas chromatograph interfaced to a Finnigan MAT TSQ 700 mass spectrometer. The GC separations were done on a fused silica column (DB-5, 15 m × 0.32 mm × 0.25 μm film thickness; J&W Scientific, Folsom, CA), programmed from 50 to 250°C at 20°C/min after a 1-min hold at 50°C. The oven temperature was held at 250°C for 10 min before programming the temperature to 300°C at 20°C/min. Helium was the carrier gas with a linear velocity of 50 cm/s in the splitless mode. The mass spectrometer was in the electron impact ionization mode at an electron energy of 70 eV, an ion source temperature of 150°C, and scanning from 40 to 650 atomic mass units at 0.5-s intervals.

RESULTS

DNA and amino acid sequence of the conserved *ERG28* gene

Hughes et al. (2) demonstrated by microarray analyses that the *ERG28* transcript is induced in *erg11, erg2, erg3*, and WT strains treated with azole antifungals. Furthermore, the overall pattern of transcriptional regulation of the *ERG28* transcript relative to the *ERG26* and *ERG27* transcripts showed correlations in more than 300 experiments of 0.843 and 0.835, respectively. Only *ERG5* and *ERG9* had slightly higher correlation coefficients ($r = 0.853$) to *ERG28* from among the 6326 yeast genes analyzed.

The *sERG28* DNA sequence encodes a 148-amino acid sequence, and homologs to this gene have been observed in *Schizosaccharomyces pombe, Arabidopsis*, and in humans. In humans, it is found to be highly expressed in adult testis and in several cancer lines (17). **Figure 1** demonstrates the homology between these conserved sequences from *Saccharomyces, Sch. pombe*, humans, and *Arabidopsis thaliana*.

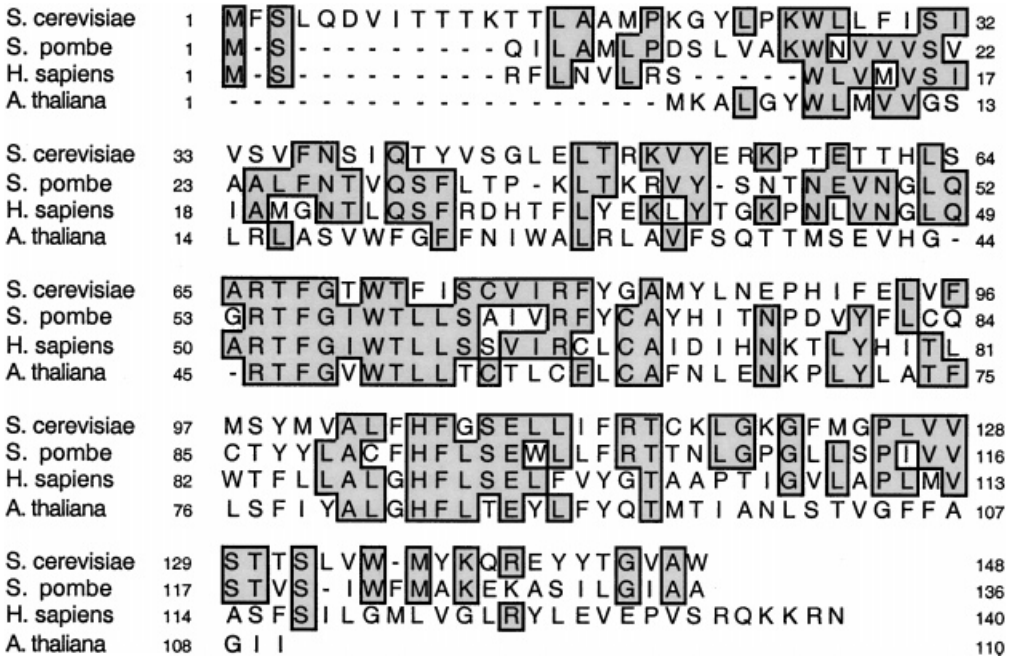

Fig. 1. Alignment of the amino acid sequences encoded by the *ERG28* gene from *S. cerevisiae, Sch. pombe, Homo sapiens*, and *A. thaliana*. Shaded areas indicate regions of sequence identity. The alignment was done with Genetics Computer Group (CGG) Wisconsin package version 9.1. Accession numbers for the *Sch. pombe*, human, and *Arabidopsis* versions of *ERG28* are T40262, NP 009107, and T00622, respectively.

Between *Sch. pombe* and *S. cerevisiae* there is 40% sequence identity and 81% sequence similarity, between *S. cerevisiae* and humans there is 29% identity and 64% sequence similarity, and between *S. cerevisiae* and *A. thaliana* there is 25% identity and 61% sequence similarity. The two yeast proteins contain at least one transmembrane domain, *Arabidopsis* contains at least two, and the human protein contains at least three based on the Kyte-Doolittle (18) algorithm of 19 amino acids and a threshold of 1.6. Interestingly, only the human protein contains a KKXX Golgi-to-ER retrieval signal at the carboxyl-terminal end (19). This amino acid sequence appears in the *Saccharomyces* and human C-4 sterol methyl oxidase proteins.

GC-MS analysis of *erg28*

Nonsaponifiable sterol analyses using GC-MS previously indicated that a number of novel sterols accumulated in the *erg28*-disrupted strain relative to the WT (2), but none of these sterols were identified. The sterol fraction from the *erg28* disruptant, isolated as described in Materials and Methods, was subject to thin-layer chromatography with CH_2Cl_2 as solvent. A polar fraction representing CAS derivatized with diazomethane had an R_f of ~0.05 followed by several fractions containing free 3-hydroxysterols and 3-keto sterols (R_f = 0.22 to R_f = 0.35). Quantifiable GC analyses of the free sterols, 3-keto sterols, and CAS sterols for the WT and *erg28* deletion strains are given in **Table 1** along with relative retention times. Total sterols for both the WT and *erg28* mutant were similar: 1.823 and 1.596 mg/g dry weight. However, no 3-keto sterols or CAS were found in the WT strain whereas in *erg28* only 38.3% of total sterols were 3-hydroxysterols and 36.2% and 25.5% were 3-keto sterols and CAS, respectively (also see **Table 2**). Table 1 also indicates that in the 3-hydroxysterol fraction, ERG comprised 66% of the total in WT and 71% of the total in *erg28*. The observation that a yeast sterol strain

TABLE 1. Distribution of free hydroxy, 3-keto, and carboxylic acid sterols in *erg28* mutant and wild-type strains

Type Sterol Molecule	RRT[a]	*ERG28*	*erg28*
3-Hydroxysterols			
Zymosterol	1.04	121	10
Fecosterol	1.079	88	70
Ergosta-5,7-dien-3β-ol	1.096	137	0
Episterol	1.099	132	0
Ergosterol	1.05	1,212	437
Lanosterol	1.14	83	37
4,4-Dimethyl-zymosterol	1.145	50	16
Unidentified sterol, MW = 396	1.119	0	20
Unidentified sterol, MW = 398	1.124	0	22
Total 3-hydroxysterols		1,823	612
3-keto sterols			
4-Methyl-zymosterone	1.09	0	274
4-Methyl-fecosterone	1.11	0	117
4-Methyl-episterone	1.176	0	48
Fecosterone	1.10	0	138
Total 3-keto sterols		0	577
Carboxylic acid sterols[b]			
4-Carboxyl-cholesta-8,24-dien-3β-ol	1.246	0	109
4-Carboxyl-fecosterol	1.29	0	170
4-Carboxyl-episterol	1.31	0	128
Total carboxylic acid sterols		0	407
Total sterols, (μg/g dry weight)		1,823	1,596

[a] RRT, Retention time on gas chromatography relative to cholesterol.
[b] Carboxylic acid sterols were derivatized and detected as methyl esters.

TABLE 2. Influence of the *erg28* disruption on the accumulation pattern of free and esterified sterols

	ERG28		*erg28*	
Sterol Class	Free Sterols	Esterified	Free Sterols	Esterified
3-Hydroxysterols	79	21	36.5	1.9
Ergosterol	(59.5)[a]	(7)	(26.8)	(0.6)
Ergosterol precursors	(19.5)	(14)	(9.7)	(1.3)
3-Keto sterols	0	0	36.2	0
Carboxylic acid sterols	0	0	23.3	2.2
Total sterols	79	21	96	4.1

[a] Numbers in parentheses reflect the breakdown of ergosterol versus accumulated ergosterol precursors.

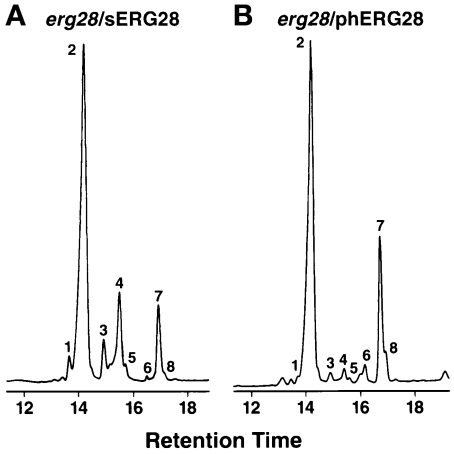

Fig. 2. GC sterol accumulation profile in an *erg28* strain complemented with a plasmid containing the *sERG28* gene (A) or the h*ERG28* gene (B). Peak 1, zymosterol; peak 2, ergosterol; peak 3, fecosterol; peak 4, ergosta-5,7-dien-3β-ol; peak 5, episterol; peak 6, 4-methylfecosterol; peak 7, lanosterol; peak 8, 4,4-dimethyzymosterol.

can simultaneously accumulate 3-hydroxysterols, 3-keto sterols, and CAS in significant quantities and still not be auxotrophic is suggestive of a partial block in the steps involved in C-4 demethylation. We previously demonstrated that *erg26* mutants accumulate CAS and *erg27* mutants accumulated 3-keto sterols but were unable to demonstrate accumulation of all three types of sterol in any C-4 demethylation mutant.

Table 2 illustrates the breakdown of free and esterified sterols in the WT and *erg28* mutant. In the WT only 3-hydroxysterols accumulate, with 79% of the sterols free and 21% esterified. Free ERG comprises 59.5% of total ERG. Whereas ERG comprises 66.5% of the total sterol in WT, it accounts for only 27.4% of the total sterol in *erg28*. This may account for the observation that this strain is slow growing but not auxotrophic. Only a small percentage of the total sterol in *erg28* is esterified, which is consistent with the observation that esterification occurs when free sterol is in excess with regard to the cell's requirement for membrane sterol (20). However, a small percentage of the total CAS is also esterified. Last, *erg28* accumulates small amounts of two 3-hydroxysterols that could not be identified.

Complementation of the *Saccharomyces erg28* strain with *sERG28* and h*ERG28* genes

pIU1600, an integrating vector containing the *sERG28* gene, was transformed into *erg28* by integrating the plasmid at the *ura3-52* locus. A high copy number plasmid (pDW394) containing the PCR-amplified h*ERG28* gene was similarly transformed into *erg28* and the sterol profiles for both are shown in **Fig. 2**. The sterol profile from the *S. cerevisiae* complementing strain (Fig. 2A) gave a nearly WT sterol profile, in which the largest peak was ERG (peak 2) with significant amounts of zymosterol (peak 1), fecosterol (peak 3), ergosta-5,7-dien-3β-ol (peak 4), episterol (peak 5), lanosterol (peak 6), and 4,4-dimethyzymosterol (peak 7) accumulating. While no 3-keto sterols were detected, increased levels of WT sterol intermediates relative to ERG were observed. These results are consistent with complementation profiles of other *Saccharomyces* ERG genes such as *erg2* and *erg3* (21, 22). The sterol complementation profile using the human gene again gave similar results, but significantly more lanosterol and 4,4-dimethylymosterol accumulated relative to ERG. In addition, a novel sterol, 4-methylfecosterol, which normally does not accumulate in WT *Saccharomyces* strains, was identified (Fig. 2B, peak 8).

DISCUSSION

Because the enzymes required for sterol biosynthesis (beginning with squalene synthase) are localized to the ER, it is not surprising that many have transmembrane-spanning (TM) domains. Among the five enzymes that do not contain such domains, Erg26p, Erg27p, Erg1p, Erg6p, and Erg7p, the latter three are found both in the ER and in cytoplasmic lipid particles (23). However, Erg26p and Erg27p, also lacking TM domains, are found exclusively in the ER and it is likely that the role of Erg28p (which contains a TM domain) may be to facilitate protein-protein interactions between the Erg26 dehydrogenase and the Erg27 3-keto reductase and/or to tether these enzymes to the ER. The advantage of having a protein that facilitates the complete demethylation of C-4 sterols would be to prevent accumulation of oxygenated C-4 intermediates (carboxylic acid and keto sterols), which may be toxic to the cell. Further, 3-keto intermediates are not esterifiable and CAS, while esterifiable, may be poor substrates for the esterification enzymes (Table 2). Whatever the exact role of Erg28p may be, as an anchor or even as a regulatory protein, it appears that this protein is conserved in sterol biosynthetic pathways from yeast to humans.

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