Molecular Cloning and Characterization of Two Isoforms of Saccharomyces cerevisiae Acyl-CoA:Steryl Acyltransferase

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Esterification of cholesterol by acyl-CoA:cholesterol acyltransferase (ACAT) is a key element in maintaining cholesterol homeostasis in cells of higher animals. In the budding yeast, Saccharomyces cerevisiae, accumulation of ergosterol esters accompanies entry into stationary phase and sporulation. We have determined that two genes in yeast, SAT1 and SAT2, encode isoenzymes of acyl-CoA:sterol acyltransferase (ASAT) which are functionally related to ACAT. The SAT1 isoform is the major catalytic isoform, accounting for at least 65–75% of total ASAT activity. Targeted deletions of one or both genes do not compromise mitotic cell growth or spore germination. However, diploids that are homozygous for a SAT1 null mutation exhibit significantly reduced sporulation efficiency. Furthermore, a larger fraction of the sporulating diploids arrest after the first meiotic division. Human ACAT expressed in sat1 sat2 mutant cells can catalyze esterification of cholesterol and, to a lesser extent, ergosterol in vitro, but restores ergosterol oleate formation in vivo to only ~8% of that catalyzed by yeast ASAT in wild-type cells.

In cells of higher animals, cholesterol required for the plasma membrane is obtained either from an exogenous source by receptor-mediated endocytosis of low density lipoprotein or by intracellular synthesis (1). Production of mevalonate, the key precursor to cholesterol and a vast number of nonsterol isoprenoids, whose functions include protein glycosylation and key precursor to cholesterol and non-sterol polyprenoid synthesis. Two isoforms of HMG-CoA reductase, which are functionally related to ACAT. The SAT1 and SAT2 genes in yeast suggest important, though as-yet unexplored, roles for sterol esters in yeast cell growth and metabolism. Further, structural and functional conservation between the yeast ASAT isoforms and mammalian ACAT was examined by expressing human ACAT in yeast cells harboring disruptions of the chromosomal copies of both SAT genes.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—Yeast strains used in this study are listed in Table I. Cultures were grown at 30 °C in rich (YPD) medium or minimal (SD; 0.67% yeast nitrogen base without amino acids) medium containing 2% glucose (8). Solid media contained 2% agar. As needed, nutritional supplements were added to a final concentration of 30 μg/ml. Cell culture density was determined by optical density measurement (9) or hemocytometer.

Synchronous sporulation in liquid medium at 30 °C was performed as described (8) and ascus formation was monitored for 15 days. Asci began to appear after 2 days and reached a maximum after 8–10 days. The number of two-spore and four-spore ascis formed after 10 days in sporulation medium were counted using a phase-contrast microscope. No difference in sporulation efficiency was observed when sporulation occurred in liquid medium or on solid media containing 2% agar. Yeast cells accumulate sterol esters and other neutral lipids as cytosolic lipid droplets (4, 5). Steryl ester accumulation and elevated acyl-coenzyme A:sterol acyltransferase (ASAT) activity is most evident in stationary-phase (6) and sporulating cells (7). Nonetheless, the requirement for sterol esters in yeast cells is poorly understood. To gain a better understanding of the role and regulation of ACAT in yeast and to address comparative structure-function issues vis-à-vis its mammalian counterpart, we sought to clone and characterize the yeast ACAT gene. As described here, the existence of two isoforms of ACAT (encoded by SAT1 and SAT2) in yeast suggests important, though apparently not essential, roles for sterol esters in yeast cell vegetative growth and metabolism. Further, structural and functional conservation between the yeast ACAT isoforms and mammalian ACAT was examined by expressing human ACAT in yeast cells harboring disruptions of the chromosomal copies of both SAT genes.

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+ The abbreviations used are: HMG, 3-hydroxy-3-methylglutaryl; ACAT, acyl-coenzyme A cholesterol acyltransferase; hACAT, human acyl-coenzyme A cholesterol acyltransferase; ASAT, acyl-coenzyme A:sterol acyltransferase; CHO, Chinese hamster ovary; bp, base pair(s); kb, kilobase(s); ORF, open reading frame; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CI 976, 2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)dodecanamide; Dup 128, N’-(2,4-difluorophenyl)-N-[5-(4,5-diphenyl-1H-imidazol-2-ylthio)pentyl]-N-heptylurea.

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Two Isoforms of Yeast Acyl-CoA:Sterol Acyltransferase

| Strain     | Yeast strains used in this study | Source              |
|------------|---------------------------------|---------------------|
| YPH500     | MATα ura3-52 ade2-101 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                      | Sikorski and Hieter (22) |
| YPH501     | MATα/MATA ura3-52/ura3-52 ade2-101/ade2-101 leu2-Δ1/leu2-Δ1 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 lys2-801/lys2-801 | Sikorski and Hieter (22) |
| FY833      | MATα ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                                | This study           |
| FY834      | MATα ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                                | This study           |
| CY226      | MATα uraltrp1 ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                      | This study           |
| CY227      | MATα uraltrp1 ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                      | This study           |
| CY230      | MATα uraltrp1 ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                      | This study           |
| CY231      | MATα uraltrp1 ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                      | This study           |
| CY232      | MATα uraltrp1 ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                      | This study           |
| CY234      | MATα/MATA uraltrp1 ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                | This study           |
| CY237      | MATα/MATA uraltrp1 ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                | This study           |
| CY238      | MATα/MATA uraltrp1 ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801                | This study           |

**Arbor, MI, respectively.** Automated DNA sequencing was performed using the PRISM DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Inc.).

**In Vivo [3H]Oleate Radio labeling—** Yeast cells (5 OD<sub>650</sub> equivalents) grown at 30°C in SD medium to mid-logarithmic or stationary phase were collected and resuspended in SD at 10 OD<sub>650</sub>/ml. As required, the ACAT inhibitors CI 976 (13) or Dup 128 (14) were added from 100 frame(ORF) of YCR048 wingenomic DNA from YPH500 cells. The PCR amplification of a 2.75 kilobase (kb) DNA fragment spanning the open reading frame (ORF) of YCR048w in genomic DNA from YPH500 cells. The PCR product was cleaved with EcoRI and ligated into pBluescript II.

Two plasmids for targeted disruption of the chromosomal YCR048w locus (SAT2) by γ-integration (22) were constructed. PvuII was used to amplify a 490-bp EcoRI-BsiEI DNA fragment spanning the 5′-end of the YCR048w ORF (including the first 23 bp of the SAT1 gene and a 570-bp HindIII-EcoRI DNA fragment flanking the 3′-end of YCR048w (including the last 90 bp of the ORF). The fragments were subcloned in tandem into the YIp vectors pRS306 (22), which includes the yeast URA3 gene, and pRS303, which includes the yeast HIS3 gene. Strain CY23 was created by introducing EcoRI-linearized pRS306-sat2 into YHP501 cells and then recovering Ura<sup>+</sup> mitotic segregants formed by sporalization of YPH501. SAT2 gene disruptions in strains FY833 and FY834 (producing strains CY30 and CY26, respectively) were created by integration of EcoRI-linearized pRS303-sat2 and selecting His<sup>+</sup> prototrophs. The sat2::URA3 and sat2::HIS3 disruptions were confirmed by genomic Southern analysis using radiolabeled probes corresponding to the 1457-bp Sat1-EcoRV fragment, 1156-bp Sat1-BamHI fragment, or the 351-bp BamHI-EcoRV fragment of YCR048w.

**Cloning and Sequencing of the SAT1 Gene—** A partial genomic clone of SAT1 was isolated by shotgun cloning. CY23 (sat2::URA3) genomic DNA (100 μg) was digested to completion with BamHI and HindIII and size-fractionated by agarose gel electrophoresis. DNA fragments 3.5–4.5 kb in length were recovered and subcloned into pBluescript II. Amenip-resistant bacteria were analyzed by colony hybridization screening (21) using the SAT2 BamHI-EcoRV fragment as a probe. Fourteen of 2500 colonies hybridized to the SAT2 probe. Southern hybridization analysis of recovered plasmid DNA identified three clones with a 4- to 5-kb insert that hybridized to the SAT2 probe. Restriction mapping and Southern analysis of the three positive clones identified a ~1-kb HindIII-Nhel fragment as the region exhibiting homology with the SAT2 gene. The HindIII-Nhel fragment was excised and subcloned into pBluescript II. Partial sequencing of both strands revealed amino acid sequence homology with Sat2p and human ACAT (65 and 34% identity, respectively, over a region of 52 amino acids extending from the HindIII site).

A full-length SAT1 genomic clone was recovered from a λ-YES yeast genomic DNA library (23). Among 150,000 phage plaques screened, nine plaques hybridized with a SAT1 probe prepared using the 1096-bp HindIII-Nhel DNA fragment of the partial genomic clone. For each of the nine phage isolates, the plasmid derivative pSE937 (a CEN4-ARS1 vector) containing the genomic DNA insert was released by cre-lox excision (10). Restriction mapping and Southern analysis identified three clones predicted to carry the full length SAT1 gene and three clones predicted to contain the full-length SAT2 gene. SAT1 localized to a 3.5-kb BamHI-Nhel region of a ~6.5-kb genomic insert in pSE937-SAT1. To sequence both strands of SAT1, the BamHI-Nhel fragment was subcloned and a series of unidirectional deletions were generated and sequenced by the dye-deoxy-terminator cycle sequencing method. Construction of SAT1 and SAT2 Null Mutations—An 800-bp Nco1 (blunted)-BamHI fragment corresponding to the 5′-flanking region of SAT1 was inserted between the SpeI (blunted) and BamHI sites of pRS304, which carries the yeast TRP1 gene, forming pRS304-SAT1. A 600-bp PvuII-BamHI fragment comprising the last 12 bp of the SAT1 coding sequence, and the adjacent 3′-flanking region was subcloned into pRS304, forming pRS304-SAT1R. The ~600-bp fragment was reiso-
lated by digestion with EcoRI and BamHI and inserted into pRS304-SAT1, forming pRS304-Δsat1.

Strains CYY31 and CYY27 were created by transforming FY833 and FY834, respectively, to Trp⁺ prototrophy with BamHI-linearized pRS304-Δsat1. The haploid sat1 sat2 null mutant, CYY28, was produced by mating CYY30 (MATa sat2::HIS3) to CYY27 (MATα sat1::TRP1). His⁺ Trp⁺ diploids were selected, sporulated, and meiotic segregants exhibiting both His⁺ and Trp⁺ growth were selected for further analysis. CYY37, CYY38, and CYY34, diploid strains that are homozygous for the sat1, sat2, and sat1 sat2 mutations, respectively, were created by mating isogenic haploids.

Expression of Human ACAT in Yeast—A variant of the human ACAT cDNA K1, in which the start codon was mutated to GTA (coding for Val) to create a unique cDNA K1, in which the start codon was mutated to CTA (coding for Leu) in the coding region of the yeast ASAT (hACAT) cDNA clone (K1) and its functional expression in insect Sf9 cells (28). Recently, a partial cDNA for rabbit ACAT (hACAT) cDNA clone (K1) and its functional expression in ACAT-deficient Chinese hamster ovary (CHO) cells (27) and in ACAT-deficient wild-type CHO cells (26). A genomic DNA fragment spanning YCR048w was obtained by genomic Southern analysis of wild-type and sat2 DNA with a SAT2 probe. Genomic DNA from YPH500 cells (lanes 1 and 3 in panels A and B) and a sat2::URA3 derivative of YPH500 (lanes 2, 4, and 5 in panels A and B) was cleaved with HindIII (lanes 1 and 2 in panels A and B), BamHI (lanes 3 and 4 in panels A and B), or HindIII plus BamHI (lanes 5 in panels A and B). DNA fragments electrophoresed through a 0.8% agarose gel and transferred to a Nytran membrane were probed with a 32P-radiolabeled SaI-EcoRV SAT2 DNA fragment and washed under high stringency (panel A) or low stringency (panel B) conditions, as described under “Experimental Procedures.” DNA fragment size (in kb) is indicated at the left.

RESULTS AND DISCUSSION

Chang et al. previously reported the isolation of a human ACAT (hACAT) cDNA clone (K1) and its functional expression in ACAT-deficient Chinese hamster ovary (CHO) cells (27) and in ACAT-deficient wild-type CHO cells (28). Recently, a partial cDNA for rabbit ACAT and full-length cDNAs for mouse and hamster ACAT have been cloned (29–31). To examine the structural and functional conservation between mammalian ACAT and its yeast counterpart, we identified and cloned a yeast genomic DNA fragment encoding the major catalytic isoform of ASAT. If YCR048w encodes yeast ASAT, then a counterpart, we identified and cloned a yeast genomic DNA fragment encoding the major catalytic isoform of ASAT. However, measurements of ASAT enzyme activity in homogenates of wild-type cells (data not shown).

To explore the possibility that a second YCR048w-related gene encoded ASAT, we reexamined the Southern blot of wild-type and sat2 genomic DNA. Incubation with the SAT2 probe under conditions of low stringency hybridization revealed an additional DNA fragment in wild-type genomic DNA that was larger than the sole fragment detected at high stringency (Fig. 1B, lanes 1 and 2). Consistent with the possible existence of two distinct, but related, ASAT genes in yeast, the SAT2 probe detected only the smaller fragment in sat2 genomic DNA (Fig. 1B, lanes 2 and 4). To isolate the SAT2-related gene, the ~4-kb fragment generated by digestion of sat2 genomic DNA with HindIII and BamHI (Fig. 1B, lane 5) was cloned, and bacterial transformants carrying the correct recombinant plasmid were identified by colony hybridization with the SAT2 probe. A ~1-kb HindIII-NsiI fragment within the ~4-kb insert hybridized to the SAT2 probe. When used to probe a genomic Southern blot under high stringency conditions, the ~1-kb HindIII-NsiI fragment detected a 5.5-kb HindIII fragment in wild-type and sat2 genomic DNA, but failed to hybridize to the 6-kb HindIII fragment containing SAT2 in wild-type genomic

FIG. 1. High stringency (A) and low stringency (B) genomic Southern analysis of wild-type and sat2 DNA with a SAT2 probe. Genomic DNA from YPH500 cells (lanes 1 and 3 in panels A and B) and a sat2::URA3 derivative of YPH500 (lanes 2, 4, and 5 in panels A and B) was cleaved with HindIII (lanes 1 and 2 in panels A and B), BamHI (lanes 3 and 4 in panels A and B), or HindIII plus BamHI (lanes 5 in panels A and B). DNA fragments electrophoresed through a 0.8% agarose gel and transferred to a Nytran membrane were probed with a 32P-radiolabeled SaI-EcoRV SAT2 DNA fragment and washed under high stringency (panel A) or low stringency (panel B) conditions, as described under “Experimental Procedures.” DNA fragment size (in kb) is indicated at the left.

FIG. 2. ASAT activity measured in cell-free extracts (A and C) or intact yeast cells (B). Panel A, ASAT-specific activity in cell-free extracts of wild-type (dark gray bar), sat1 (open bar), sat2 (black bar), and sat1 sat2 (light gray bar) cells grown to mid-logarithmic phase in SD and assayed as described under “Experimental Procedures.” Panel B, ASAT activity in intact cells grown to mid-logarithmic phase in SD, pulse-radiolabeled with [3H]oleate for 15 min at 30°C, and analyzed by TLC as described under “Experimental Procedures.” Panel C, ASAT-specific activity in cell-free extracts of wild-type and mutant cells harboring pSE937 lacking a genomic DNA insert (YCp), pSE937-SAT1 containing the SAT1 gene (pSAT1), or pSE937-SAT2 containing the SAT2 gene (pSAT2) assayed as described under “Experimental Procedures.”
DNA (data not shown). Partial sequencing of both strands of the 1-kb Hin III-Nsi I segment revealed 65% amino acid identity with SAT2 and 34% amino acid identity with the hACAT cDNA over a region of 52 amino acids. The Hin III-Nsi I fragment was then used as a probe to isolate a full-length genomic clone of the SAT2-related gene (hereafter referred to as SAT1) from a λ-YES yeast genomic DNA library (23).

To determine the effect of deleting the SAT1 gene, sat1::TRP1 and sat1::TRP1 sat2::HIS3 null mutants were created (described under “Experimental Procedures”). Disruption of the SAT2 gene did not alter ASAT activity. By comparison, ASAT enzyme activity in sat1 cells decreased to 25% of the activity in wild-type cells (Fig. 2 A) and in vivo synthesis of ergosterol oleate by sat1 cells was reduced to 35% of wild-type cells (Fig. 2 B). Importantly, neither assay detected ASAT activity in sat1 sat2 cells. These results demonstrate that SAT1 and SAT2 encode two isoforms of ASAT. Furthermore, SAT1 encodes the major ASAT catalytic isoform. Assuming Sat2p contributes no more ASAT activity to wild-type cells than to sat1 cells, then the SAT1 isoform accounts for at least 75% of ASAT activity. Similar contributions by hMG1 and hMG2 to HMG-CoA reductase activity were reported: hmg1 and hmg2 cells exhibited 17% of wild-type activity, whereas hmg2 cells exhibited >100% of wild-type activity (32). Whereas both hmg1 cells and hmg2 cells were viable, viable HMG-CoA reductase double null mutants were not recovered (32). In contrast, both haploid and homozygous diploid sat1 sat2 mutants were viable, as were cells carrying only a single mutation at the SAT1 or SAT2 locus. Thus, under the growth conditions tested, failure to synthesize sterol esters does not impair cell growth.

To confirm that genomic clones of SAT1 and SAT2 isolated from the λ-YES yeast genomic DNA library code for functionally distinct isoforms of ASAT, rescue of ASAT enzyme activity in singly and doubly mutant yeast cells was examined. Introduction of pSE936-SAT1 into sat1 or sat1 sat2 cells restored ASAT activity to wild-type levels. Transformation of sat1 sat2 cells with pSE936-SAT2, by contrast, yielded only a partial rescue of ASAT activity which was comparable to the activity measured in sat1 cells. Of interest, neither plasmid significantly increased ASAT activity in wild-type cells. The ability of pSE936-SAT1 to restore wild-type levels of activity to sat1 sat2, but not to enhance ASAT activity in wild-type cells suggests that ASAT activity is tightly regulated to prevent overaccumulation of steryl esters.

Further support for the identity of SAT1 and SAT2 as the structural genes for ASAT comes from analysis of the predicted SAT1 and SAT2 proteins. Fig. 3 shows the alignment of the deduced amino acid sequences of SAT1 (642 amino acids), SAT2 (610 amino acids), and hACAT (550 amino acids). Overall amino acid identity between the SAT1 and SAT2 isoforms is...
43%. Human ACAT shares 17% amino acid identity with the SAT1 isozyme and 16% identity with the SAT2 isozyme. A hydrophilic domain in Sat1p and Sat2p (located at amino acids 513–553 in Sat1p) exhibits 50% identity with hACAT. Within this domain, a tyrosine kinase phosphorylation motif (RX-RX-RX-RX-S-P) located at amino acids 517–524 in Sat1p (underlined in Fig. 3) is conserved in the yeast, human, hamster, and mouse enzymes. This region will be a target for future studies of the mechanism and regulation of ACAT and ASAT activity. Moreover, the sequence M-K-X-H-S-F (amino acids 396–341 in Sat1p), containing a serine residue (boxed in Fig. 3) shown to be necessary for hamster ACAT activity (31), is conserved in the yeast and mammalian enzymes. In mammalian cells, ACAT is an integral membrane protein of the endoplasmic reticulum (12, 34). Similarly, ASAT activity has been reported to co-purify with the microsome fraction of yeast (35). Chang et al. (27) reported two potential transmembrane α-helices in hACAT, as well as several other regions that are very hydrophobic but unlikely to span the endoplasmic reticulum membrane. Kyte-Doolittle hydropathy analysis (36) revealed hydrophobic regions in Sat1p and Sat2p corresponding in position to those in hACAT. There are six potential N-linked glycosylation sites in Sat1p, two of which are conserved in Sat2p.

In contrast to the normal vegetative cell growth phenotype of sat1 and sat1 sat2 cells, examination of sporulating diploids revealed that a null mutation in SAT1, alone or in combination with a SAT2 null mutation, affected sporulation efficiency. Samples of three independent liquid sporulation cultures of sat1 sat1 sat2 diploids in liquid sporulation medium. By comparison, free sterol increased only 14-fold in the presence of 1% Me2SO. Me2SO alone (+) or 200 μM CI 976 (+). Panel B, ASAT activity in intact cells grown to mid-logarithmic phase in SD, preincubated for 30 min with 1% Me2SO alone (+) or 200 μM CI 976 (+), and pulse-radiolabeled with [14C]oleate for 15 min at 30°C in the presence of 1% Me2SO (+) or 200 μM CI 976 (+). Lipid extraction and TLC were performed as described under “Experimental Procedures.”

ACAT from various mammalian cells and human ACAT expressed in Sf9 insect cells are inhibited both in vivo and in vitro by the ACAT inhibitors CI 976 and Dup 128 (28). To test functional conservation between hACAT and the ASAT enzyme isoforms, the effectiveness of CI 976 and Dup 128 in blocking ester synthesis and sporulation. ACAT from various mammalian cells and human ACAT expressed in Sf9 insect cells are inhibited both in vivo and in vitro by the ACAT inhibitors CI 976 and Dup 128 (28). To test functional conservation between hACAT and the ASAT enzyme isoforms, the effectiveness of CI 976 and Dup 128 in blocking ergosterol ester formation in wild-type and mutant cells was examined. Measurements of wild-type cell extracts showed that 100 μM CI 976 inhibited 90% of ASAT activity (data not shown) and that >95% inhibition occurred in the presence of 200 μM CI 976 (Fig. 5A). Equivalent inhibition of human ACAT expressed in Sf9 cells was reported at ~50 μM CI 976. Dup 128

**FIG. 4.** Sporulation efficiency of wild-type and homozygous sat1, sat2, and sat1 sat2 diploids in liquid sporulation medium.

Three isolates of each genotype were grown and sporulation induced as described under “Experimental Procedures.” The total number of cells (including two- and four-spore asci) and the number of two- and four-spore asci in a sample of each culture were determined under a phase-contrast microscope. The results for each genotype are plotted as the percentage of total number of cells counted (mean ± S.E., n = 3) that were two-spore asci (diads) or four-spore asci (tetrad). Multivariate analysis of variance was performed to determine the significance of sporulation differences among the wild-type and mutant strains. For two-spore asci, F8,10 = 7.56, p = <0.005; for four-spore asci, F8,10 = 123.27, p = <0.0001; for two-spore plus four-spore asci, F8,10 = 15.28, p = <0.0001.

**FIG. 5.** Inactivation of ASAT activity in cell-free extracts (A) or intact yeast cells (B) by ACAT inhibitor CI 976. Panel A, ASAT-specific activity in cell-free extracts of wild-type (dark gray bar), sat1 (light gray bar), and sat2 (black bar) cells grown to mid-logarithmic phase in SD and assayed as described under “Experimental Procedures” following addition of 1% Me2SO alone (−) or 200 μM CI 976 (+). Panel B, ASAT activity in intact cells grown to mid-logarithmic phase in SD, preincubated for 30 min with 1% Me2SO alone (−) or 200 μM CI 976 (+), and pulse-radiolabeled with [14C]oleate for 15 min at 30°C in the presence of 1% Me2SO (−) or 200 μM CI 976 (+). Lipid extraction and TLC were performed as described under “Experimental Procedures.”

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Two Isoforms of Yeast Acyl-CoA:sterol Acyltransferase

Expression of hACAT cDNA in wild-type and sat1 sat2 yeast cells. Panel A, Immunodetection of hACAT expressed in Sf9 insect cells infected with recombinant baculovirus containing hACAT cDNA K1 (lane 1) and in wild-type (lanes 2 and 3) or sat1 sat2 yeast cells (lanes 4 and 5) harboring pCY6 (hACAT fused in-frame behind the influenza hemagglutinin epitope in pAD54) (lanes 2 and 4) or pCY7 (hACAT fused out-of-frame behind the influenza hemagglutinin epitope in pAD54) (lanes 3 and 5). SDS-PAGE and immunoblotting with affinity-purified anti-hACAT were performed as described under “Experimental Procedures.” The arrow on the left indicates the 50 kDa form of hACAT produced in Sf9 cells. The arrowhead on the right indicates the ~52-kDa influenza hemagglutinin-tagged hACAT produced in yeast cells. The lower molecular mass form of influenza hemagglutinin-tagged hACAT detected in yeast cell extracts (lanes 2 and 4) is probably a proteolytic breakdown product of the 52-kDa form; a similar observation was reported when hACAT was overexpressed in CHO cells (12). Panel B, ASAT activity in intact wild-type cells (dark grey bar), wild-type cells harboring pCY6 (light grey bar), sat1 sat2 cells (black bar), and sat1 sat2 cells harboring pCY6 (open bar) grown to mid-logarithmic phase in SD, pulse-radiolabeled with [3H]oleate for 15 min at 30°C, and analyzed by TLC as described under “Experimental Procedures.” Panel C, ASAT-specific activity in cholesterol-reconstituted (black bar) or ergosterol-reconstituted (grey bar) lysates of hACAT-expressing sat1 sat2 cells grown to mid-logarithmic phase in SD and assayed as described under “Experimental Procedures.”

was less effective, inhibiting ~50% of ASAT activity at 30–40 μM (data not shown). ASAT activity in sat1 and sat2 cell extracts was inhibited >95% by 200 μM CI 976 (Fig. 5A). By contrast, inhibition of ergosterol-[3H]oleate formation in vivo was incomplete. In the presence of 200 μM CI 976, incorporation of radioactivity into ergosterol ester was reduced by ~75–80% in wild-type, sat1, and sat2 cells (Fig. 5B). CI 976 and Dup 128 are fatty acid analogues that inhibit the active site of ACAT (38). The sensitivity of both yeast ASAT isozymes to CI 976 in the in vitro enzyme assay suggests that the fatty acid binding domain and/or catalytic domain of hACAT is conserved in yeast ASAT.

To further test the functional similarity of hACAT and the yeast ASAT isozymes, we quantified the activity in vivo and in vitro of hACAT produced in wild-type and sat1 sat2 yeast cells. To express hACAT in yeast cells, the coding sequence of a derivative of the hACAT cDNA pK1 was inserted into pAD54 (24) downstream of and in frame with the coding sequence for the influenza hemagglutinin epitope. Expression of hACAT was demonstrated by immunodetection of SDS-PAGE fractionated yeast cell extracts. As reported previously (12, 28), anti-hACAT IgG detected two species of hACAT, with apparent molecular masses of 50 kDa (arrow) and 56 kDa, produced in insect SF9 cells (Fig. 6A, lane 1). Human ACAT synthesized in wild-type yeast cells (lane 2) and sat1 sat2 cells (lane 4) migrated with an apparent molecular mass of 52 kDa, which is consistent with the presence of the 18-amino acid influenza hemagglutinin epitope. No anti-hACAT reactive protein was detected in extracts prepared from wild-type cells (lane 2) or sat1 sat2 cells (lane 5) harboring pCY7, in which hACAT is not in frame with the influenza hemagglutinin epitope. Expression of hACAT in wild-type cells did not increase [3H]oleate incorporation in vivo into ergosterol esters (Fig. 6B). However, synthesis of hACAT in sat1 sat2 cells increased formation of ergosteryl-[3H]oleate from background level (0.6% of wild-type incorporation) to ~8% of that measured in wild-type cells. Low hACAT activity in vivo could result from formation of inactive hACAT or could reflect hACAT specificity for cholesterol. To distinguish between these possibilities, we measured the activity of hACAT made in sat1 sat2 cells in vitro using a reconstituted assay to which exogenous cholesterol or ergosterol was added. Measurements performed in the presence of cholesterol liposomes demonstrated that hACAT produced in yeast had a specific activity of ~900 pmol/min/mg (Fig. 6C). Cheng et al. (28) reported that the specific activity of hACAT in extracts of SF9 cells 48 h after infection with recombinant baculovirus is at least 10 times higher than that detected in...
human cell lines. The specific activity of hACAT expressed in yeast was ~70% of the activity measured in S9 cells. When yeast-expressed hACAT activity was measured in the presence of ergosterol liposomes, specific activity was reduced more than 80%. Thus, it appears that hACAT synthesized by yeast cells is active but unable to esterify ergosterol efficiently.

Identification of two structural genes in yeast, SAT1 and SAT2, coding for conserved isoforms of ASAT raises several issues concerning the function and regulation of ASAT that will be addressed in future studies. Perhaps the most intriguing question focuses on the presence of two SAT isoforms. Our data demonstrate that disruption of both SAT genes does not impair vegetative cell growth under standard laboratory cell culture conditions. By contrast to contraregulation of HMG1 and HMG2 by oxygen (3), which finely tunes availability of oxygen-independent non-sterol derivatives of mevalonate and oxygen-dependent sterol products, there is no obvious rationale for oxygen regulation of ASAT expression; ASAT substrates are products of squalene epoxidation formed only during aerobic growth. Nonetheless, a study of sterol esterification in yeast hem mutants provided evidence for a link between heme competency and ASAT activity (39). Moreover, examination of a hem1 hap1 mutant suggested the presence of two enzymes with ASAT activity, one of which might be HAP1 regulated (39).

Consistent with a model for differential regulation of two SAT isoforms was the demonstration that sterol substrate specificity changed as cells entered stationary growth phase (40). Whereas cells in exponential growth esterify ergosterol precursors (lacking Δ2 or Δ24 double bonds or the Δ24-methyl group), cells in stationary phase esterify ergosterol predominantly (40, 41). Thus, Sat1p and Sat2p may represent functionally related isoforms with different sterol substrate specificities. To test if the activities of Sat1p and Sat2p change when cells enter stationary phase, sterol ester synthesis in vivo was examined. Measurements of sterol ester formation in exponential and stationary phase cultures revealed that sat2 cells in exponential phase consistently showed a 5–10% increase in activity compared to wild-type cells (Fig. 7A; also Fig. 2B). A similar finding was reported for hem2 cells (32). By contrast, sat2 cells examined in stationary phase showed a ~25% reduction in sterol ester formation compared to wild-type cells (Fig. 7B).

An alternate model for control of ASAT activity and sterol substrate specificity involves the formation of hetero-oligomers composed of Sat1p and Sat2p. Cheng et al. (28) pointed out that the kinetics of hACAT activity in response to increasing the concentration of cholesterol in the reconstituted ACAT enzyme assay supported a model in which hACAT enzyme is a homodimer. Altering the stoichiometry of Sat1p and Sat2p in null mutants or as wild-type cells progress into stationary phase could modulate ASAT activity. The presence of a leucine heptad motif (42–44) between amino acids 347 and 369 in Sat1p (and conserved in Sat2p), as previously identified in hACAT (27), is consistent with the possibility of homo- or hetero-oligomerization of ASAT.

Our observation of impaired sporulation in diploid cells homozygous for the sat1 mutation provides evidence for a functional role for sterol esters during meiosis or ascospore formation. Also, modulation of isozyme activity during growth lends support to the possibility that Sat1p and Sat2p exhibit distinct substrate specificities. Both functions can be further explored in future studies. Additionally, the yeast ASAT-deficient strain will serve as a valuable host for regulation studies as well as structure-function analysis of mammalian ACAT.

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Note Added in Proof—SAT1 and SAT2 correspond to the yeast genes ARE2 and ARE1, respectively, recently reported by Yang et al. (46).
Molecular Cloning and Characterization of Two Isoforms of Saccharomyces cerevisiae Acyl-CoA:Sterol Acyltransferase
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