Identification of Two Mammalian Reductases Involved in the Two-carbon Fatty Acyl Elongation Cascade*

Young-Ah Moon‡ and Jay D. Horton‡§

From the Departments of §Molecular Genetics and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9046

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The de novo synthesis of fatty acids occurs in two distinct cellular compartments. Palmitate (16:0) is synthesized from acetyl-CoA and malonyl-CoA in the cytoplasm by the enzymes acetyl-CoA carboxylase 1 and fatty acid synthase. The synthesis of fatty acids longer than 16 carbons takes place in microsomes and utilizes malonyl-CoA as the carbon source. Each two-carbon addition requires four sequential reactions: condensation, reduction, dehydration, and a final reduction to form the elongated fatty acyl-CoA. The initial condensation reaction is the regulated and rate-controlling step in microsomal fatty acid elongation. We previously reported the cDNA cloning and characterization of a marine long chain fatty acyl elongase (LCE) (1). Overexpression of LCE in cells resulted in the enhanced addition of two-carbon units to C12-C16 fatty acids, and evidence was provided that LCE catalyzed the initial condensation reaction of long chain fatty acid elongation. The remaining three enzymes in the elongation reaction have not been identified in mammals. Here, we report the identification and characterization of two mammalian enzymes that catalyze the 3-ketoacyl-CoA and trans-2,3-enoyl-CoA reduction reactions in long and very long chain fatty acid elongation, respectively.

Ninety percent of all fatty acids present in mammalian cells are derived from de novo synthesis. The predominant fatty acids synthesized in mammals are long chain fatty acids 16–18 carbons in length. Long chain fatty acids are important components of phospholipids, represent the largest energy storage reservoir in the form of triglycerides, and are the preferred precursors of cholesterol. The highest rate of de novo fatty acid synthesis occurs in the liver, which converts excess glucose into fatty acids for storage and transport. During times of caloric excess, glucose is converted to pyruvate, which is converted to citrate in the mitochondria and transported to the cytosol where ATP citrate lyase uses citrate to produce acetyl-CoA. Acetyl-CoA is carboxylated by acetyl-CoA carboxylase 1 to form malonyl-CoA. Fatty acid synthase (FAS) then uses malonyl-CoA, acetyl-CoA, and NADPH to elongate fatty acids in two-carbon increments in the cytosol (2). The principal fatty acid produced by FAS in rodents is palmitic acid, which contains 16 carbons and is designated 16:0 (3).

The mammalian enzymes that elongate palmitic acid (16:0) and very long chain fatty acids (>C18) have been localized to the endoplasmic reticulum (ER) and are shown schematically in Fig. 1 (4). Microsomal fatty acid elongation uses malonyl-CoA as the two-carbon donor and consists of four sequential and independent reactions: 1) a condensation between a fatty acyl-CoA and malonyl-CoA to form 3-ketoacyl-CoA; 2) a reduction of the 3-ketoacyl-CoA using NADPH to form 3-hydroxyacyl-CoA; 3) a dehydration of 3-hydroxyacyl-CoA to trans-2,3-enoyl-CoA; and 4) a reduction of trans-2,3-enoyl-CoA to saturated acyl-CoA (5). Unlike the multifunctional FAS enzyme, the enzymes that carry out microsomal fatty acid elongation are encoded by separate genes.

Enzymes involved in microsomal fatty acid elongation have been characterized most extensively by genetic deletion studies in Saccharomyces cerevisiae. Three proteins, designated Elo1p, Elo2p, and Elo3p, participate in the initial condensation reaction of microsomal fatty acyl elongation. Elo1p is required for the elongation of C14 to C16 fatty acids (6), and Elo2p and Elo3p are required for the synthesis of very long chain fatty acids (7).

Six mammalian homologues of the yeast ELO enzymes have been described (Fig. 1). Like their yeast counterparts, these enzymes exhibit some fatty acid chain length substrate specificity. The first mammalian elongase identified was Cig30 (cold-induced glycoprotein of 30 kDa), which is the functional equivalent of yeast Elo2p (8). Sac1 and Sac2 (sequence similarity to Cig30 and 2) subsequently were identified based on homology to Cig30 (9). Sac1 is the functional equivalent of Elo3p in yeast (9). Definitive fatty acid substrate specificity has not been assigned to Sac2, although two of its substrates are arachidonic (20:4) and eicosapentaenoic (20:5) acids (1). ELOVL4 (elongation of very long chain fatty acids-like 4) was identified by linkage and haplotype analysis in families with two forms of autosomal dominant macular dystrophy and is expressed only in tissues with high contents of very long chain fatty acids; therefore, it is likely that ELOVL4 is involved in the elongation of very long chain fatty acids (10). HEL0 was identified based on sequence homology with yeast Elo2p and has a broad range of very long chain fatty acid substrate speci-
Fatty acyl-CoA

| Condensation | LCE | HEL01 Cig30 | SSC1 SSC2 ELOVL4 |
|--------------|-----|-------------|------------------|

3-Ketoacyl-CoA

| Reduction | KAR |
|-----------|-----|

3-Hydroxyacyl-CoA

| Dehydration | Trans-2,3-enoyl-CoA |
|-------------|---------------------|

| Reduction | TER |
|-----------|-----|

Elongated Fatty acyl-CoA

**Experimental Procedures**

**Materials**—[2-14C]Malonyl-CoA (40–60 mCi/mmol) was obtained from PerkinElmer Life Sciences. Redivue [α-32P]dCTP (3000 Ci/mmol) was obtained from Amersham Biosciences. Palmitoyl-CoA, palmitic acid (16:0), γ-linolenic acid (18:3ω-6), arachidonic acid (20:4ω-6), eicosapentaenoic acid (20:5ω-3), fatty acid-free BSA, coenzyme A, sodium salt, ATP, NADPH, malonyl-CoA, and monoclonal anti-HA antibody (clone number F-7; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were purchased from the manufacturer.

**Cloning of Mamalian 3-Ketoacyl-CoA and trans-2,3-Enoyl-CoA Reductase cDNAs and Construction of Expression Plasmids—cDNAs encoding the putative human and mouse microsomal 3-ketoacyl-CoA reductase (KAR) were identified by a BlastP search of the NCBI database using the S. cerevisiae protein Ybr159p. Human and mouse cDNAs (GenBank™ accession numbers NM_016142 and AF064635, respectively) were identified that encode proteins that are ~31% identical to the yeast 3-ketoacyl-CoA reductase protein Ybr159p. The trans-2,3-enoyl-CoA reductase (TER) proteins were identified by a BlastP search using the S. cerevisiae protein Tsc13p, which encodes the yeast trans-2,3-enoyl-CoA reductase. Human and mouse cDNAs (GenBank™ accession numbers AA52373 and AK010984, respectively) were identified that encode proteins that are ~34% identical to Tsc13p.

**In Vitro Fatty Acid Elongation Assay—** Palmitoyl-CoA or BSA-bound fatty acids were used as substrates for all reactions. BSA-bound fatty acids were prepared as 5 or 10 mM solutions as described (1). The assays contained 0.02 mg of microsomal protein in 50 mM potassium phosphate, pH 6.5, 5 μM retinene 20, 150 μM [2-14C]Malonyl-CoA (6.5 dpm/pmol), 1 mM NADPH, 20 μM BSA in a final reaction volume of 0.2 ml. For assays using BSA-bound fatty acids, the reaction mixtures contained 0.05 mg of microsomal protein in 50 mM potassium phosphate, pH 6.5, 5 μM retinene 20, 20 μM BSA-bound fatty acid, 0.05 μM coenzyme A, 1 mM ATP, 1 mM MgCl₂, 150 μM [2-14C]Malonyl-CoA (6.5 dpm/pmol), and 1 mM NADPH in a final volume of 0.2 ml. To initiate the elongation reaction, 0.05 mg of microsomal protein from transfected cells was added, and the incubation was continued for the indicated times. The reactions were stopped by adding 0.1 ml of 75% methanol at 0 °C for 10 min. After three washes with PBS, the cells were incubated for 1 h at 4 °C in 1% BSA in PBS (buffer A). The cells were then incubated in buffer A at 4 °C with a mouse monoclonal HA antibody (HA probe F-7; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (20 μg/ml) and a rabbit anti-calnexin polyclonal antibody (StressGen Biotechnologies Corp., Victoria, Canada) (1:200 dilution) for 1 h. The cells were washed three times with buffer B (0.1% BSA in PBS), and primary antibodies were localized by incubating the cells for 1 h at room temperature in buffer A containing 2 μg/ml goat anti-rabbit IgG conjugated to Alexa Fluor 568 and goat anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR). After incubation with the secondary antibody, the cells were washed three times with buffer B, quickly rinsed with PBS and distilled water, and analyzed with a Leica TCS SP confocal microscope (Leica Microsystems Inc., Heidelberg, Germany).

**Immunofluorescence Microscopy—** Chinese hamster ovary K1 cells (ATCC CCL-61) were set up on glass coverslips in 6-well plates at a density of 1.0 × 10⁵/well in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 5% fetal calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (day 0). On day 1, 0.5 μg of indicated plasmids was transfected using 3 μl of FuGENE 6 (Roche Molecular Biochemicals) in serum-free Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium. On day 3, the cells were washed with PBS and then fixed and permeabilized by incubating in 2 ml of methanol at -20 °C for 10 min. After three washes with PBS, the cells were incubated for 1 h at 4 °C in 1% BSA in PBS (buffer A). The cells were then incubated in buffer A at 4 °C with a mouse monoclonal HA antibody (HA probe F-7; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (20 μg/ml) and a rabbit anti-calnexin polyclonal antibody (StressGen Biotechnologies Corp., Victoria, Canada) (1:200 dilution) for 1 h. The cells were washed three times with buffer B (0.1% BSA in PBS), and primary antibodies were localized by incubating the cells for 1 h at room temperature in buffer A containing 2 μg/ml goat anti-rabbit IgG conjugated to Alexa Fluor 568 and goat anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR). After incubation with the secondary antibody, the cells were washed three times with buffer B, quickly rinsed with PBS and distilled water, and analyzed with a Leica TCS SP confocal microscope (Leica Microsystems Inc., Heidelberg, Germany).
KOH (w/v) and 0.2 ml of ethanol, saponified at 70 °C for 1 h, and then acidified by adding 0.4 ml of 5 N HCl with 0.2 ml of ethanol. Fatty acids were collected in three independent extractions using 1 ml of hexane. The extractions were pooled, dried under nitrogen, and separated by TLC using hexane/diethyl ether/acetic acid (30:70:1) as described (1). The TLC plates were exposed to a PhosphorImager screen, the resulting image was analyzed, and the lipids were quantified using a Bio-Imaging Analyzer with BAS1000 MacBAS 2.1 software (Fuji Medical Systems, Stamford, CT).

**RNAi-mediated Inhibition of KAR and TER—Double-stranded (ds) RNA oligonucleotides were synthesized by Dharmacco Research (Lafayette, CO) for human KAR, TER, and an irrelevant control gene, vesicular stomatitis virus glycoprotein. The oligonucleotide sequences are listed in Table 1. On day 0, HeLa cells (ATCC CCL-2) or HepG2 (ATCC HB-8065) cells were set up at a density of 4 × 10⁵ cells/60-mm dish. HeLa cells were cultured in minimum essential medium supplemented with 10% fetal calf serum, 1× nonessential amino acid mix (Cellgro, Herndon, VA), 1 nm sodium pyruvate, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate, respectively. dsRNAs (0.2 μg) were transfected on days 1, 2, and 3 using OligofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. On day 4, the cells were harvested for membrane protein and total RNA as described (1).**

**Quantitative Real Time PCR**—total RNA was extracted from the indicated tissues of C57BL/6J mice using TRIzol (Life Technologies, Grand Island, NY). The cDNA was synthesized from 1 μg of total RNA using MuLV reverse transcriptase (Life Technologies) and oligo(dT) primers according to the manufacturer’s instructions. Total RNA was subjected to Northern blot analysis using 32P-labeled mouse cDNA probes for human 3-Ketoacyl-CoA and trans-2,3-Enoyl-CoA Reductases

**Table 1**

| Human gene                      | Primer sequence                  | GenBank accession |
|---------------------------------|----------------------------------|-------------------|
| 3-Ketoacyl-CoA reductase        | 5′-UTCCGAAGCCCAAACUUUGGATT-3′    | NM_016142         |
| trans-2,3-Enoyl-CoA reductase   | 5′-TTAGGCCCUGGUGGAAACCCU-3′      | AF222742          |
| Vesicular stomatitis virus glycoprotein | 5′-GGCUUAAACAGACAGGGUTT-3′  | M35207            |
The putative human and mouse TER proteins were identified by a BlastP search of the NCBI database using the yeast Tsc13p. The identified human (GenBank™ accession number AF222742) and mouse (GenBank™ accession number AK010984) cDNAs encode proteins that are 33 and 34% identical to that of Tsc13p (14). The putative human trans-2,3-enoyl-CoA reductase cDNA has an in-frame stop codon 21 nucleotides prior to the initiation methionine. The translational reading frames of the human and mouse putative TER cDNAs predict proteins 308 amino acids in length. The overall identity of the human and mouse TER proteins is 95%.

An alignment of the yeast, mouse, and human TER amino acid sequences is shown in Fig. 3A. Unlike the KAR proteins, no consensus ER retention motif is present in the mouse or human TER sequence. Hydropathy analysis using the Kyte and Doolittle algorithm (22) predicts the presence of as many as five transmembrane domains (Fig. 3B). No mitochondrial or peroxisomal targeting sequences were identified in these proteins. The tissue expression pattern of TER was determined using human and mouse multiple tissue Northern blots as described for KAR (Fig. 3C). A single ~1.2-kb mRNA was identified by Northern blotting in all of the tissues tested from human and mouse (Fig. 3C). The tissue expression of TER essentially mirrored that of KAR.

To determine whether KAR or TER participated in microsomal fatty acyl elongation, expression vectors utilizing the CMV promoter were assembled that encoded the human KAR or TER proteins with HA epitope tags at their N terminus. HEK-293 cells were transfected with the human KAR or TER expression plasmids, and cytosolic and microsomal proteins were
FIG. 4. Subcellular localization of KAR, TER, and LCE in Chinese hamster ovary K1 cells. Chinese hamster ovary K1 cells were grown on coverslips and transfected with pCMV-Script (A–C), pCMV-HA-KAR (D–F), pCMV-HA-TER (G–I), or pCMV-HA-LCE (J–L) as indicated. The cells were fixed and immunostained with antibodies to the HA tag of the expressed proteins (A, D, G, and J) and to an endogenous ER membrane protein, calnexin (B, E, H, and K), as described under “Experimental Procedures.” The merged images (C, F, I, and L) show the co-localization of the expressed proteins and calnexin.

separated by SDS-PAGE to determine the subcellular localization of the proteins. As predicted from the hydrophathy profiles, immunoblot analysis using an anti-HA antibody revealed that the expressed KAR and TER proteins were present only in the microsomal fraction (data not shown). To study the subcellular localization of these proteins directly, we performed double-label immunofluorescence studies of the HA epitope-tagged KAR, TER, or LCE proteins that were transfected in Chinese hamster ovary K1 cells (Fig. 4). Staining with the anti-HA antibody revealed that KAR and TER co-localized with the ER resident protein calnexin and the condensing enzyme LCE (Fig. 4, C, F, I, and L). Additional stains for a mitochondrial protein, Grp75, showed no significant co-localization with KAR, TER, or LCE, and stains for a cis-compartment Golgi resident protein, GM130, showed no co-localization with KAR and LCE (data not shown). A small degree of co-localization of GM130 and TER was found, the significance of which is not known.

Previously, we showed that the elongation of palmitoyl-CoA (16:0) was increased significantly in microsomes from HEK-293 cells transfected with the condensing enzyme, LCE (1). Elongation activity was determined by measuring the amount of 14C incorporated from [2-14C]malonyl-CoA into elongated fatty acid products. LCE overexpression markedly enhanced the initial condensation of palmitoyl-CoA to 3-ketostearoyl-CoA. The LCE-mediated increase in palmitoyl-CoA condensation caused the subsequent reactions to become rate-limiting, leading to the accumulation of elongation intermediates, which could be separated and identified by TLC (1). The accumulation of elongation intermediates provided a tool to study the potential function of the KAR and TER proteins. Working under the hypothesis that KAR functions as a long chain 3-ketoacyl-CoA reductase, the co-expression of KAR with LCE in cells should result in the selective disappearance of 3-ketostearoyl-CoA intermediate in microsomes incubated with palmitoyl-CoA. Similarly, the co-expression of TER with LCE should result in the selective disappearance of the trans-2,3-stearoyl-CoA intermediate if the TER protein functions as a trans-2,3-enoyl-CoA reductase.

Fig. 5 (lanes 4–6 and 10–12) shows that the overexpression of LCE alone resulted in the accumulation of all of the elongation intermediates in microsomes from HEK-293 cells incubated with palmitoyl-CoA as the fatty acid substrate. Co-expression of LCE and human KAR resulted in the selective disappearance of 3-ketostearoyl-CoA, suggesting that the KAR protein enhanced the reduction of 3-ketostearoyl-CoA to 3-hydroxystearoyl-CoA (Fig. 5, lanes 7–9). Similarly, co-expression of LCE and human TER resulted in the disappearance of the trans-2,3-stearoyl-CoA intermediate (Fig. 5, lanes 13–15). This result suggested that TER functions to reduce trans-2,3-stearoyl-CoA to stearoyl-CoA. A duplicate set of experiments was performed using the mouse orthologues of KAR and TER, and similar results were obtained (data not shown).

To determine whether KAR exhibits fatty acid substrate specificity, RNAi was employed to selectively reduce the expression of KAR in cultured cells. Inhibiting KAR expression should result in the accumulation of the 3-ketoacyl-CoA intermediate if the fatty acid tested is a substrate of the enzyme. HeLa cells were transfected with the indicated dsRNAs and microsomal protein, and the total RNA was isolated (Fig. 6). The endogenous mRNA level of KAR was selectively reduced 4-fold in cells transfected with dsRNA oligonucleotides corresponding to KAR, whereas the expression of TER and cyclophilin was unchanged (Fig. 6, lower panels). Microsomes from transfected cells were incubated with long and very long chain fatty acid substrates, and the 14C-labeled elongation products from the fatty acid elongation reaction were separated by TLC (Fig. 6, upper panel). Microsomes from cells transfected with dsKAR oligonucleotides accumulated the 3-ketoacyl-CoA intermediates for all fatty acids tested in the elongation assay (Fig. 6, lanes 3, 6, 9, and 12). The final elongated fatty acyl-CoA product was reduced by 40–50% in microsomes from cells transfected with dsKAR oligonucleotides. These results supported the conclusion that KAR functioned as a 3-ketoacyl-CoA reductase and demonstrated that KAR reduced very long chain 3-ketoacyl-CoA substrates as well as long chain fatty acyl-CoAs.

A similar set of RNAi experiments was performed using dsRNAs oligonucleotides corresponding to human TER in HepG2 cells (Fig. 7). HepG2 cells were used for these experiments because the lower endogenous expression of TER apparently facilitated the inhibition of TER by RNAi. The transfection of HepG2 cells with dsTER oligonucleotides resulted in a
selective 4-fold reduction in endogenous TER mRNA levels (Fig. 7, lower panels). trans-2,3-Enoyl-CoA intermediates accumulated in microsomes from dsTER oligonucleotide transfected cells for all fatty acid substrates tested in the elongation assay (Fig. 7, lanes 3, 6, 9, and 12). The final fatty acyl-CoA product was reduced by 50–60% in the elongation assay with all of the fatty acids tested. Together, the data from these overexpression and inhibition studies suggested that TER functioned as a trans-2,3-enoyl-CoA reductase and that TER reduced long and very long chain fatty acid trans-2,3-enoyl-CoA substrates.

All of the previously identified enzymes required for long chain fatty acid biosynthesis are regulated by the sterol regulatory element-binding protein (SREBP) family of transcription factors (23). The SREBP family members are designated SREBP-1a, SREBP-1c, and SREBP-2. The SREBP-1 isoforms preferentially activate genes encoding fatty acid biosynthetic enzymes, whereas SREBP-2 preferentially activates genes specifying cholesterol biosynthetic enzymes. To be active, SREBPs undergo two sequential cleavages that require three proteases...
designated S1P and S2P (24). All three proteins are required for normal SREBP activation inasmuch as the deletion of any one results in the absence of all transcriptionally active forms of SREBPs (24).

To determine whether KAR and TER mRNA levels were regulated in a manner similar to other fatty acid biosynthetic genes, the mRNA levels of FAS, LCE, KAR, and TER were measured in livers from mice that either overexpress the transcriptionally active forms of SREBPs or that lack all SREBP isoforms as a result of inactivating SCAP (17, 19, 20). Consistent with previous studies, the mRNA levels of FAS and LCE were increased ~20-fold in livers from SREBP-1a transgenic mice (TgSREBP-1a) (Table II) (1, 17). SREBP-2 overexpression (TgSREBP-2) also increased the expression of FAS and LCE mRNAs, but to a lesser extent than the overexpression of SREBP-1a. Conversely, removing all transcriptionally active forms of SREBPs by deleting SCAP in liver (SCAP−/−) resulted in a 4-fold decrease in FAS expression and a ~2-fold reduction in LCE mRNA. The mRNAs for KAR and TER were largely unaffected, either by SREBP overexpression or by the absence of SREBPs. These data suggest that unlike other enzymes required for fatty acid biosynthesis, KAR and TER mRNA levels are not regulated by SREBPs in vivo.

**DISCUSSION**

In the current studies, we identified two mammalian reductases that participate in the microsomal elongation of long and very long chain fatty acids. BlastP searches of the NCBI data bases identified human and mouse homologues of the *S. cerevisiae* proteins Ybr159p, a 3-ketoacyl-CoA reductase, and Tsc13p, a *trans*-2,3-enoyl-CoA reductase. Biochemical studies of the recombinant human and mouse proteins confirmed that they exhibit KAR and *trans*-2,3-enoyl-CoA reductase activities. The enzymes responsible for microsomal fatty acyl elongation have not been purified previously. Therefore, the genes identified in this study provide an initial molecular characterization of the reductases that carry out the second and fourth steps in microsomal long and very long chain fatty acyl elongation in mammals.

The 3-ketoacyl-CoA reductase, KAR, identified in this study shares sequence similarity with members of the short chain dehydrogenase superfamily (25), which are characterized by a nucleotide co-factor-binding region (Rossmann-fold) and an active site that consists of a triad of catalytically important and highly conserved Ser-Tyr-Lys residues. KAR is expressed in all tissues, with the highest levels of expression occurring in tissues that are directly involved in lipid metabolism. We provide evidence that KAR is a 3-ketoacyl-CoA reductase and that it represents the second enzyme in the microsomal fatty acyl two-carbon elongation cascade. Whether KAR is the only enzyme that can carry out the 3-ketoacyl-CoA reduction in cells could not be addressed in the current studies. In *S. cerevisiae*, the majority of very long chain fatty acyl 3-ketoacyl-CoA activity is due to Ybr159p; however, the genetic disruption of the YBR159c gene does not completely abolish all 3-ketoacyl-CoA reductase activity (12). The ybr159 mutants are viable but have a slowed rate of growth. The residual 3-ketoacyl-CoA reductase activity in the *ybr159* mutants was attributed to a gene that encodes 1-acyldihydroxyacetone-phosphate reductase (13). A mammalian gene has not been identified; therefore it was not possible to test whether a mammalian orthologue of *AYR1* could mediate the reduction of 3-ketoacyl-CoAs.

The *trans*-2,3-enoyl-CoA reductase, TER, is 32% identical to the yeast *trans*-2,3-enoyl-CoA reductase, Tsc13p. Kohlwein et al. (14) identified and characterized the yeast Tsc13 protein as a *trans*-2,3-enoyl-CoA reductase and reported that it belonged to an evolutionarily conserved family of proteins present.
in all mammals, yeast, and Arabidopsis thaliana. The human and mouse \textit{trans}-2,3-enoacyl-CoA reductase proteins are also \textasciitilde97\% identical to the rat SC2 protein that was originally identified in a screen for cDNAs that encoded synapic glycoproteins (27). The \textit{trans}-2,3-enoacyl-CoA reductase family members share sequence similarity with steroid 5a-reductase, an ER enzyme that catalyzes the reduction of testosterone to dihydrotosterone (27, 28). Human TER and steroid 5a-reductase are \textasciitilde30\% identical and 45\% similar over the C-terminal \textasciitilde130 amino acids. Neither protein contains classic NADPH-binding sites; however, at least eight amino acid residues at the C-terminal end of steroid 5a-reductase type 2 are crucial for NADPH binding (29). Four of these eight residues are conserved in the yeast and mammalian TER proteins. Therefore, although the identified TER protein does not contain a classic NADPH-binding site, the sequence similarity with steroid 5a-reductase suggests that it utilizes NADPH as a co-factor.

The overexpression and inhibition of TER in cultured cells demonstrated that the enzyme is capable of mediating the \textit{trans}-2,3-enoacyl-CoA reduction of both long and very long chain fatty acids. Inhibition of TER by RNAi resulted in the marked accumulation of \textit{trans}-2,3-enoacyl-CoA substrate intermediate for all fatty acids tested (Fig. 7). A small amount of the preceding 3-hydroxyacyl-CoA intermediate also accumulated in microsomes from \textit{dsTER} oligonucleotide transfected cells. This 3-hydroxyacyl-CoA intermediate is a substrate for the dehydratase enzyme. Although the dehydratase protein has not been identified, Knoll et al. (30) have shown that the dehydratase reaction in microsomal fatty acid elongation is reversible. Therefore, the inhibition of TER could result in the accumulation of the 3-hydroxyacyl-CoA intermediate as a consequence of the reverse reaction. These results do not preclude the possibility that TER may participate in the dehydratase reaction in addition to catalyzing the fourth and final step in the microsomal fatty acyl elongation cascade.

Studies in yeast and mammals have demonstrated that microsomal fatty acyl-CoA condensing enzymes exhibit fatty acyl chain length specificity (1, 7, 9, 10). It has been suggested that the post-condensation enzymes do not exhibit carbon chain length specificity (4). The current studies provide support for this hypothesis. Although all possible fatty acid substrates could not be tested, the data of Fig. 6 show that inhibition of KAR resulted in the accumulation of the 3-ketoacyl-CoA substrate for palmitic (16:0), \( \gamma \)-linolenic (18:3\-n-6), arachidonic (20:4\-n-6), and eicosapentaenoic (0:5n-3) fatty acids. Similar results were obtained using myristic (14:0), palmitoleic (16:1), docosatetraenoic (22:4\-n-6), and docosapentaenoic (22:5n-3) fatty acids as substrates in the microsomal elongation assay described in Fig. 6 (data not shown). TER inhibition by RNAi resulted in the accumulation of the \textit{trans}-2,3-enoacyl-CoA intermediates for the same broad range of fatty acid substrates identified as KAR substrates. Despite this accumulation, it is possible that other unidentified 3-ketoacyl-CoA or \textit{trans}-2,3-enoacyl-CoA reductases have greater activities for a given fatty acyl substrate than those characterized in the current studies. The current data demonstrate that the identified KAR and TER do not exhibit the strict fatty acyl chain length substrate specificity displayed by LCE and other characterized condensing enzymes.

All known fatty acid biosynthetic enzymes isolated to date are regulated by the SREBP family of transcription factors (23). The overexpression of SREBPs in liver results in the accumulation of fatty acids that are 18 carbons in length, because of the activation of FAS and LCE (1, 23, 31). Reducing SREBP levels by eliminating the SCAP protein in liver resulted in a 40–70\% reduction in the mRNA levels of all fatty acid biosynthetic genes (20). In contrast to other lipogenic genes, the mRNA levels of KAR and TER were largely unaffected by SREBP expression levels in liver (Table II).

Lipogenesis is hormonally regulated by insulin, and the ability of this hormone to stimulate lipogenesis is mediated by SREBP-1c. The results from the transgenic and knockout mice would suggest that KAR and TER are not regulated by insulin in a manner similar to other lipogenic genes (32, 33). The activities of the four microsomal elongation enzymes previously have been measured under conditions of high and low insulin (34). These studies demonstrated that only the initial condensation reaction catalyzed by LCE is regulated by insulin. The mRNA levels of the identified genes responsible for these reactions follow a similar pattern of regulation. The condensing enzyme, LCE, is suppressed in livers of fasted mice (low insulin) and increased more than 20-fold in liver from mice that were fasted and refed a high carbohydrate diet (high insulin) (35). In similar fasting and refeeding studies, the mRNA levels of TER and KAR remain unchanged in mouse liver (data not shown). Together, the \textit{in vivo} data support the hypothesis that KAR and TER are constitutively expressed and that the initial condensation reaction is the regulated step in microsomal fatty acyl elongation.

In summary, the overexpression and inhibition of the human KAR and TER in cultured cells demonstrate that they function as 3-ketoacyl-CoA and \textit{trans}-2,3-enoacyl-CoA reductases, respectively. The lack of any measurable fatty acid carbon chain length substrate specificity for either KAR or TER suggests that the six known condensing enzymes channel the fatty acyl intermediates to a common series of enzymes that produce the elongated fatty acyl-CoA product (Fig. 1). Whether KAR and TER are essential for the long and very long chain fatty acyl elongation \textit{in vivo} or whether other proteins also possess 3-ketoacyl-CoA and \textit{trans}-2,3-enoacyl-CoA activities will require analysis in knockout mice.

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