Identification and molecular characterization of acyl-CoA synthetase in human erythrocytes and erythroid precursors

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Full-length cDNA species encoding two forms of acyl-CoA synthetase from a K-562 human erythroleukaemic cell line were cloned, sequenced and expressed. The first form, named long-chain acyl-CoA synthetase 5 (LACS5), was found to be a novel, unreported, human acyl-CoA synthetase with high similarity to rat brain ACS2 (91 % identical). The second form (66 % identical with LACS5) was 97 % identical with human liver LACS1. The LACS5 gene encodes a highly expressed 2.9 kb mRNA transcript in human haemopoietic stem cells from cord blood, bone marrow, reticulocytes and fetal blood cells derived from fetal liver. An additional 6.3 kb transcript is also found in these erythrocyte precursors; 2.9 and 9.6 kb transcripts of LACS5 are found in human brain, but transcripts are virtually absent from human heart, kidney, liver, lung, pancreas, spleen and skeletal muscle. The 78 kDa expressed LACS5 protein used the long-chain fatty acids palmitic acid, oleic acid and arachidonic acid as substrates. Antibodies directed against LACS5 cross-reacted with erythrocyte membranes. We conclude that early erythrocyte precursors express at least two different forms of acyl-CoA synthetase and that LACS5 is present in mature erythrocyte plasma membranes.

Key words: fatty acid, K-562 cells, LACS5, plasma membrane, red-cell membrane.

INTRODUCTION

Long-chain acyl-CoA synthetase (LACS, EC 6.2.1.3) has a key role in erythrocyte membrane fatty acyl metabolism. Although the erythrocyte lacks lipid synthesis *de novo*, the fatty acyl groups of the phospholipids are continuously renewed. Acyl-CoA, the product of acyl-CoA synthetase, is a crucial intermediate in the de-acylation/re-acylation phospholipid remodelling, which was first identified by Lands [1]. Acyl-CoA lysophospholipid acyltransferase uses both acyl-CoA species and lysophospholipid to generate phospholipid [2,3]. In addition, acyl-CoA in the erythrocyte is used for the acylation of membrane proteins [4] and as substrate by carnitine acyltransferase [5]. Acyl-CoA synthetases form an enzyme family whose members seem to differ in substrate specificity, subcellular localization and tissue distribution. Members of this family are found in a wide variety of tissues and cell organelles. They are essential in endoplasmic reticulum for glycerolipid synthesis, in mitochondria and peroxisomes for fatty acid β-oxidation, and in plasma membranes for re-acylation reactions. In the human, four forms of LACS have been identified. LACS1 [6] and LACS2 [7] from human liver have a high similarity (96 % amino acid identity), but are distinctly different from LACS3 [8] and LACS4 [9,10] from human placenta and brain. These enzymes exhibit molecular masses of 75–80 kDa. LACS1 and LACS2 have been identified on chromosome 4 [7], LACS3 on chromosome 2 [8] and LACS4 on the X chromosome [9]. Although both platelets [11] and erythrocytes [12] exhibit LACS activity, no information is available on the protein structure of these enzymes in the peripheral blood or precursors of these cells, in human or other mammalian systems. The reported human forms of LACS have a relatively high similarity to their counterparts in the rat. Human LACS1 is similar to rat liver ACS1 [13], human LACS3 to rat ACS3 [14] and human LACS4 to the rat ACS4, which is found in rat adrenal glands, ovary and testis [15]. A common theme in these enzymes is the presence of domains with high similarity to enzymes of the luciferase family [14–16]. We report a new human LACS from a K-562 human erythroleukaemic cell line that we identify as LACS5, which is distinctly different from human LACS1, LACS2, LACS3 and LACS4. The enzyme uses long-chain fatty acids as substrate, and the mRNA for this protein is highly expressed in erythrocyte precursors and human brain, but is practically absent from other human tissues including lung, liver, kidney, heart and muscle.

MATERIALS AND METHODS

Materials

Unless indicated otherwise, all restriction enzymes, recombinant *Taq* DNA polymerase and T4 DNA polymerase enzymes were purchased from Promega Corp. (Madison, WI, U.S.A.), and the cDNA synthesis components were from Gibco BRL (Gaithersburg, MD, U.S.A.). The QIAprep Spin Miniprep, Quiquick PCR purification kit, QIAX II Agarose Gel Extraction Kit and PCR Kit were purchased from Qiagen (Valencia, CA, U.S.A.). The radioisotope [α-^{35}S]dATP (1000 Ci/mmol) was obtained from DuPont–NEN Research Products (Boston, MA, U.S.A.); [α-^{32}P]dCTP (3000 Ci/ml) was from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Oligonucleotides were synthesized and purified by HPLC at 0.5 mmol scale by Operon Technologies (Alameda, CA, U.S.A.). Frozen pellets of K-562 cells (chronic myelogeneous leukaemia cells in terminal blast crisis) and CD34$^+$ cells were gifts from Dr. Dan Eaton (Genentech, San Francisco, CA, U.S.A.). The samples of human bone marrow, cord blood and reticulocytes were obtained from Dr. Lori Styles (Children's Hospital Oakland, Oakland, CA, U.S.A.). Human fetal liver samples for the isolation of fetal blood cells were obtained from a non-profit research tissue bank (Advanced Bioscience Resources, Alameda, CA, U.S.A.) in accordance with the guidelines of the Department of Health and Human Service Regulations. Palmitic acid, oleic acid, arachidonic acid, ATP and CoA

Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; LACS, long-chain acyl-CoA synthetase.

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were purchased from Sigma (St. Louis, MO, U.S.A.). The radiolabelled [^{14}C]-palmitic acid, -oleic acid and -arachidonic acid were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.).

General recombinant DNA methods

Standard molecular biology techniques were performed essentially as described by Sambrook et al. [17]. All PCR amplification was performed on a GeneAmp® PCR System 2400 from Perkin Elmer Corp. (Foster City, CA, U.S.A.).

Reverse-transcriptase-mediated PCR

Total RNA was isolated from 7-day-old and 14-day-old CD34$^+$ cells, K-562 cells, fetal blood cells [18], reticulocytes, cord blood, peripheral blood and bone marrow with an RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.) in accordance with the protocol of the manufacturer. The first-strand cDNA template from K-562 total RNA was synthesized by mixing 3.2 μl of diethyl pyrocarbonate-treated water, 3 μl of DNase-treated RNA (300 ng) and 5 μl of 10 μM oligo(dT) primer. The reaction mixtures were heated for 5 min at 70 °C. After snap-cooling on ice, the reaction was continued in a total volume of 20 μl containing 4 μl of 5 × first-strand cDNA synthesis buffer, 2 μl of 100 mM dithiothreitol, 0.8 μl of 0.5 mM dNTP mix, 0.5 μl of 40 i.u./μl RNasin (ribonuclease inhibitor) and 1.5 μl (300 standard EC units) of Superscript RNase H^{-ve} reverse transcriptase (Gibco). The reaction mixtures were incubated at 37 °C for 90 min, followed by 10 min at 75 °C. The cDNA (3 μl) was amplified by PCR with two sets of degenerate primers derived from the most highly conserved amino acid sequences of LACS1, LACS2, ACS2, ACS3 and ACS4 [6,7,14–16]. The first amino acid sequence chosen, TSG(T/S)TG(N/L/R)PKG(A/V)M, was represented by the PCR1 primer 5′-CAC(G/A)AG(C/T)GG-(T/A)(A/T)C(A/T/C)ACAGG(G/C/A)A(A/T)(C/T)C-C(C/A)AA(A/G)GG(T/A)G(C/T)(G/A/C)ATG-3′, and the amino acid sequence LKIIDRKK(H/D)(I/L)(F/V)K was used to design the anti-sense PCR2 primer 5′-GTTT(C/A)A(C/A)-(A/T)A(G/T)(A/G)T(C/G)CTTTTT(A/C)CG(G/A)TC(A/G)AT(A/G)AT(C/T)TT(G/C/A)AG-3′. The third amino acid sequence chosen, PT(V/L/I)(F/M)(P/A)(V/A)VPRLLN, was represented by the PCR3 primer 5′-CCCAC(T/G/A/C)(G/C/A)T(C/G/T)(T/A)T(C/G)(C/G)C(C/A/T)G(T/C)(G/T)-GT(T/C)CC(A/G)(A/G/C)(G/A)A(C/A)T(G/C)(C/A)-T G(A/G)AC-3′; the anti-sense PCR4 primer 5′-(G/A/T)GG-(C/A/G/T)(T/A)(G/C/A)(G/A/C)(A/C)ATT(C/T)TCCAA-T(A/G)TC(T/A/G/C)CC(A/T)GTG(C/T)(A/G)(A/C/T)-A(G/A)CCA-3′ corresponded to the amino acid sequence WL(H/C)TGDIG(K/E)(W/F)(L/E/H)P. PCR amplification was performed with each primer at 2.5 μM in a total volume of 50 μl with other components from the PCR kit in accordance with Qiagen's protocol, with the use of Q solution. The amplification was done at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1.5 min, followed by a final extension at 72 °C for 10 min. The PCR products were separated on 1.5 % (w/v) agarose, gel-purified, subcloned in a TA-TOPO cloning vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced.

Sequencing

Partial sequences for all clones were obtained by the dideoxy chain-termination method with the Sequenase Version 2.0 DNA Sequencing Kit (US Biochemical, Cleveland, OH, U.S.A.) by using T7 DNA polymerase and [α-^{35}S]dCTP. The sequence of the entire cDNA species was determined in both directions on a Standard Li-Cor Semi Automated Sequencing Instrument by MacConell Research Corporation (San Diego, CA, U.S.A.). Nucleotide and amino acid sequences were compared with the non-redundant sequence databases present at the National Center for Biotechnology Information (NCBI) by using the BLAST network service [19].

Isolation of full-length cDNA species

By using sequences from both ends of the PCR products, two sets of gene specific primers were designed: GSP 1 (anti-sense), 5′-AGTTTAAATATATGCTTTTTCCGATCAATAATTTTAA-3′; GSP 2 (sense), 5′-CACAAGCGGCACGACAGGGAACC-CAAAAGGTGCGATG-3′; GSP 3 (anti-sense), 5′-TGGTAA-CCATTTTCCTATGTCCCCTGTGTGTACCA-3′; and GSP 4 (sense), 5′-CCCACTGTCTTCCCCGTGGTTCCAAGACTG-CTGAAC-3′.

By using primers GSP1 and GSP2 and the adaptor primer −1 (AP1; Clontech Laboratories, Palo Alto, CA, U.S.A.), two products of rapid amplification of cDNA ends ('RACE') were amplified from Human Leukaemia, Chronic Myelogenous Marathon-Ready™ cDNA (Clontech). The amplification was done at 94 °C for 3 min and at 80 °C for 1 min, followed by 28 cycles of 94 °C for 10 s, 60 °C for 3 min and 72 °C for 3 min, with a final extension at 72 °C for 7 min, in accordance with the manufacturer's protocol. The 5′ (1.8 kb) and 3′ (1.3 kb) products overlapped for 860 bp. Similarly, by using primers GSP3 and GSP4, 5′ (1.6 kb) and 3′ (1.0 kb) products were amplified that overlapped by 562 bp. The sequences obtained for all four PCR products were compared with known sequences for other acyl-CoA synthetases. Finally, two full-length cDNA species were generated by PCR, separated on 1.5 % (w/v) agarose, gel-purified, subcloned into a TA vector and sequenced entirely.

Cloning of LACS5 into the pET28a expression vector

A product encoding the entire open reading frame of LACS5 cDNA (from bp 115 to bp 2094 of the nucleotide sequence) was amplified by PCR with the use of gene-specific forward and reverse primers with *Nhe*I and *Hin*dIII restriction sites. The forward primer sequence used was 5′-CTGTCAGCTAGC-ATGCAGACACAGGAG-3′ and the reverse primer was 5′-TTTCCTAAGCTTTCACATGGGGATTGAGTA-3′. Full-length cDNA, subcloned into TA-TOPO, was used as a template. The amplified fragment was resuspended in appropriate buffers, digested with *Nhe*I and *Hin*dIII and cloned into the polylinker region of pET28a vector (Novagen, Milwaukee, WI, U.S.A.) to obtain the expression plasmid LACS5/pKTM99. The clone was verified by restriction digestion for the correct insert.

Expression of LACS5

BL21 cells, a strain of *Escherichia coli*, were transformed with the LACS5/pKTM99 plasmid or the pET28a vector. Wild-type BL21 cells, BL21 cells with the pET28a vector and cells with the LACS5 expression plasmid LACS5/pKTM99 were grown in Luria–Bertani medium with or without the antibiotic kanamycin (50 μg/ml) at 37 °C with shaking until the D_{600} was between 0.6 and 1.0. In one aliquot, the addition of isopropyl β-D-thio-galactoside (IPTG) to a final concentration of 0.1 mM induced the fusion protein. To another aliquot no IPTG was added. Cultures from these aliquots were subsequently grown for 18 h at 37 °C. The cells were harvested, resuspended in lysis buffer [50 mM potassium phosphate (pH 7.4)/1 mM EDTA/1 mM

dithiothreitol/10 % (w/v) glycerol/1 mM PMSF] and sonicated at 4 °C. The proteins from the lysates were separated by SDS/PAGE and detected by staining with Coomassie Blue.

Northern blot analysis

Total RNA (10–20 μg) from cells or tissues (erythroid precursor) was dissolved in 20 μl of sample buffer [50 % (v/v) formamide/6.5 % (v/v) formaldehyde/20 mM Mops (pH 7.0)], denatured at 65 °C for 5 min and separated by electrophoresis on a 1.2 % (w/v) agarose gel containing 6.5 % (v/v) formaldehyde. RNA was transferred on a Nytran® membrane (Schleicher & Schuell) by downward transfer (Turboblotter; Schleicher & Schuell) and the membrane was cross-linked by UV at 1200 mJ/cm in a Stratalinker (Stratagene, Keene, NH, U.S.A.). Equal loading of samples was confirmed by staining the gel with ethidium bromide or hybridization of the same blot with a β-actin probe. The complete transfer was verified by staining of the membrane with Methylene Blue. Northern Territory™ human normal blotting (Clontech) was also used to check the expression in other human tissues. After prehybridization of each blot in 5 × SSC [20 × SSC is 3 M NaCl/0.3 M sodium citrate (pH 7.0)]/5 × Denhardt's solution/0.1 % SDS/200 μg/ml sonicated salmon sperm DNA for 4 h at 42 °C, membranes were hybridized with the [^{32}P]dCTP randomly labelled 860 bp fragment of LACS5 cDNA (nt 939–1799) or a 561 bp fragment of LACS1 cDNA (nt 1078–1639) as a probe. Membranes were incubated with probe (1.5 ng/ml; specific radioactivity approx. 1.5×10^9 c.p.m./μg) in 50 % (v/v) formamide/5 × SSC/5 × Denhardt's solution/0.1 % SDS/200 μg/ml sonicated salmon sperm DNA for 16 h at 42 °C. After hybridization, the blots were washed twice in 2 × SSC/0.2 % SDS for 10 min at room temperature, and twice in 0.1 × SSC/0.1 % SDS for 30 min at 55 °C, followed by a final wash in 2 × SSC for 5 min at room temperature. Blots were exposed to a Phosphor Screen (Molecular Dynamics, Sunnyvale, CA, U.S.A.) for 8 h. The expression signal was quantified with a PhosphorImager with ImageQuant 3.0 software (Molecular Dynamics).

Assay of acyl-CoA activity in cell lysates

The acyl-CoA synthetase activity was determined by measuring the formation of [1-^{14}C]acyl-CoA from 1-^{14}C-labelled fatty acid, CoA and ATP [20]. Cell lysates were prepared from a defined number of cells. The lysate equivalent of 10^8 cells was incubated for 20 min in 0.15 ml of a standard reaction mixture containing 15 μmol of Tris/HCl, pH 8.0, 1 μmol of ATP, 100 nmol of CoA, 750 nmol of dithiothreitol, 3 μmol of $MgCl_2$ and 40 μl of a 50 mM $NaHCO_3$/7.5 mM Triton X-100 solution containing either palmitic, oleic or arachidonic acid, trace labelled with the same radiolabelled fatty acid (10 nmol of fatty acid, approx. 2×10^5 d.p.m.). The reaction was stopped by the addition of 2.25 ml of a mixture of propan-2-ol/heptane/2 M sulphuric acid (40:10:1, by vol.), followed by 1.5 ml of heptane, 1 ml of water and vigorous vortexing. After centrifugation (5 min at 2000 ***g***), the upper layer was removed and the lower aqueous phase was washed twice with 2 ml of heptane containing 4 mg/ml palmitic acid, and finally with 2 ml of heptane. The radioactivity in the upper (heptane) layers and lower (aqueous) phase was determined by scintillation counting (Beckman). The radioactivities in the upper (fatty acid) and lower (fatty acyl-CoA) phase were used to calculate the enzyme activity and expressed as nmol of fatty acyl-CoA formed. The results were corrected for blanks (incubations for 20 min in the absence of cell lysates), which contained approx. 1 % of the radioactivity in the lower phase.

Antibodies against LACS5

To generate antibodies directed against LACS5, we made use of synthetic peptides for antiserum production [21]. The following peptide (single-letter code), based on the LACS5 sequence residues 71–91, was selected on the basis of antigenicity, hydrophilicity and ease of synthesis: NH_2-(GC)GSGPQLLTHYY-DDART-CO_2H. The amino acid residues in parentheses were added to facilitate conjugation of the peptide to the carrier protein (Imject Keyhole Limpet Haemocyanin; Pierce, Rockford, IL, U.S.A.). The peptide was generated by Bio-Synthesis (Lewisville, TX, U.S.A.) and rabbits were injected. Serum was collected and used in accordance with standard Western blotting protocols.

RESULTS AND DISCUSSION

Acyl-CoA synthetase catalyses the formation of acyl-CoA from fatty acid, ATP and CoA. The mature erythrocyte lacks internal membrane structures; in these cells, plasma membrane-bound acyl-CoA synthesis serves as a first step in acylation processes of plasma membrane phospholipids and proteins [4,12]. In other cells, including erythrocyte precursors, acyl-CoA synthesis is important in processes such as lipid synthesis *de novo* and β-oxidation. Several (iso)forms of this enzyme have been cloned in mammals. In rat, five forms of this enzyme have been described with different tissue expression patterns [13–16,22], and in human four forms have been described until now [6–10]. The goal of our study was to identify the acyl-CoA synthetase in erythroid cells; here we describe the cloning and expression in different tissues of a novel human erythroid acyl-CoA synthetase.

Two sets of degenerate oligonucleotide primers (PCR1 to PCR4), as indicated in the Materials and methods section, were designed on the basis of an amino acid sequence in the most conserved region of reported acyl-CoA synthetase sequences from human and rat tissues [7,14,16]. With these primers we isolated two cDNA fragments from the K562 cell line. The K562 cell line and library were chosen because this human cell line is regarded as an appropriate substitute for erythroid cells [23]. The first fragment, generated by using the primers PCR1 and PCR2, was 860 bp long; the second, from primers PCR3 and PCR4, was 676 bp long. The full-length cDNA species for both fragments were amplified from a human K-562 cDNA Marathon library, with gene-specific primers, on the basis of the fragment sequence as indicated in the Materials and methods section. The derived amino acid sequence of the first cDNA was found to be a new and previously unreported human acyl-CoA synthetase, which we designated LACS5. The second cDNA fragment isolated from the K-562 library encoded an amino acid sequence virtually identical with that reported to be present in human liver (LACS1). Figure 1 (upper panel) shows the nucleotide and predicted amino acid sequences of LACS5. The full-length cDNA obtained consisted of 2555 bp, including 114 bp at the 5′ untranslated region, followed by the 2091 bp open reading frame encoding 697 amino acid residues, and 350 bp at the 3′ untranslated region. The 5′ untranslated region of the mRNA contained an in-frame stop codon at 108 bp upstream of the predicted initiation codon ATG. Two consensus polyadenylation signals, AATAAA and ATTAAA, were located 29 and 3 bp upstream of the poly(A) tail respectively. The calculated molecular mass of LACS5 was 77.6 kDa, a value in close agreement with that determined by SDS/PAGE of the overproduced protein (shown in Figure 3, lower panel).

Acyl-CoA synthesis involves membrane-bound substrates (acyl groups) as well as compounds from the cytosol (CoA and ATP). The acyl-CoA product is subsequently used by other membrane

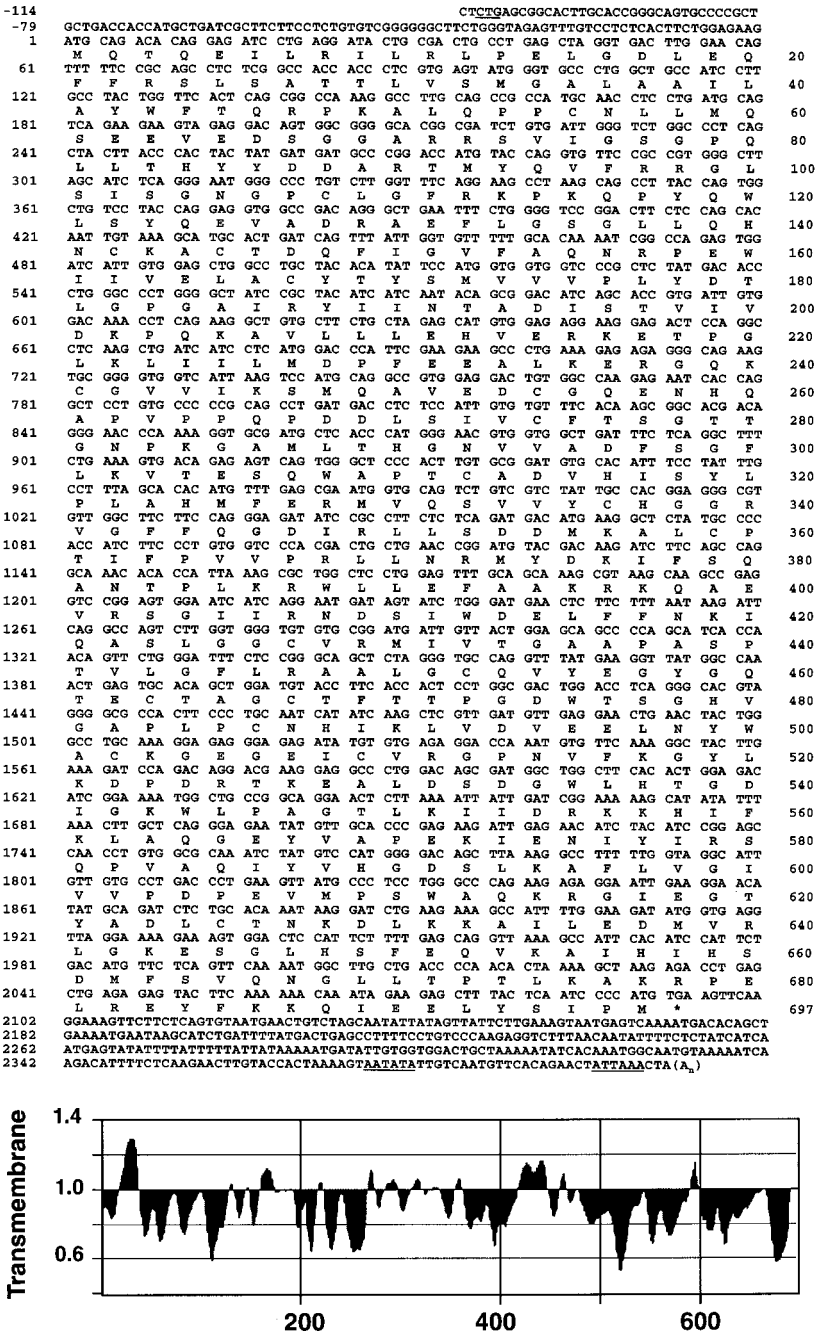


Figure 1 Amino acid analysis of LACS5

Upper panel: nucleotide and deduced amino acid sequences of human LACS5 cDNA. The deduced amino acid sequence of the predicted open reading frame is shown under the nucleotide sequence (GenBank accession number AF129166). Nucleotide residues are numbered at the left and the amino acid residues are numbered at the right. Two potential polyadenylation signals in the 3′ untranslated region are underlined. An in-frame start codon in the 5′ untranslated region is underlined. The stop codon in the 3′ region is indicated with an asterisk. Lower panel: the transmembrane region of LACS5. Predicted transmembrane sequence of LACS5 with the Argos Alogrithm in Mac Vector 6.5. The hydrophilicity is given on the basis of the amino acid sequence. Values higher than 1.0 denote hydrophobic regions that might tend to be buried inside the molecule or inside the hydrophobic membrane environment. Values below the axis denote hydrophilic regions that might be exposed on the outside of the molecule in hydrophilic environments.

bound enzymes such as acyl-CoA lysophospholipid acyltransferase. In erythrocytes these enzyme activities are associated with the inner leaflet of the plasma membrane [12]. To evaluate a possible membrane-binding sequence in LACS5, we performed a transmembrane domain prediction with Mac Vector 6.5 (Oxford Molecular System) with the use of the Argos Algorithm (Figure 1, lower panel). The prediction with this software agrees strongly with the prediction of protein localization sites with PSORT (http://psort.nibb.ac.jp/form.html), which indicated a 16-residue (residues 25–41) transmembrane helix. Most of the polypeptide (residues 42–697) is predicted to extend as a 657-residue cytoplasmic tail. These results indicate that the sequence

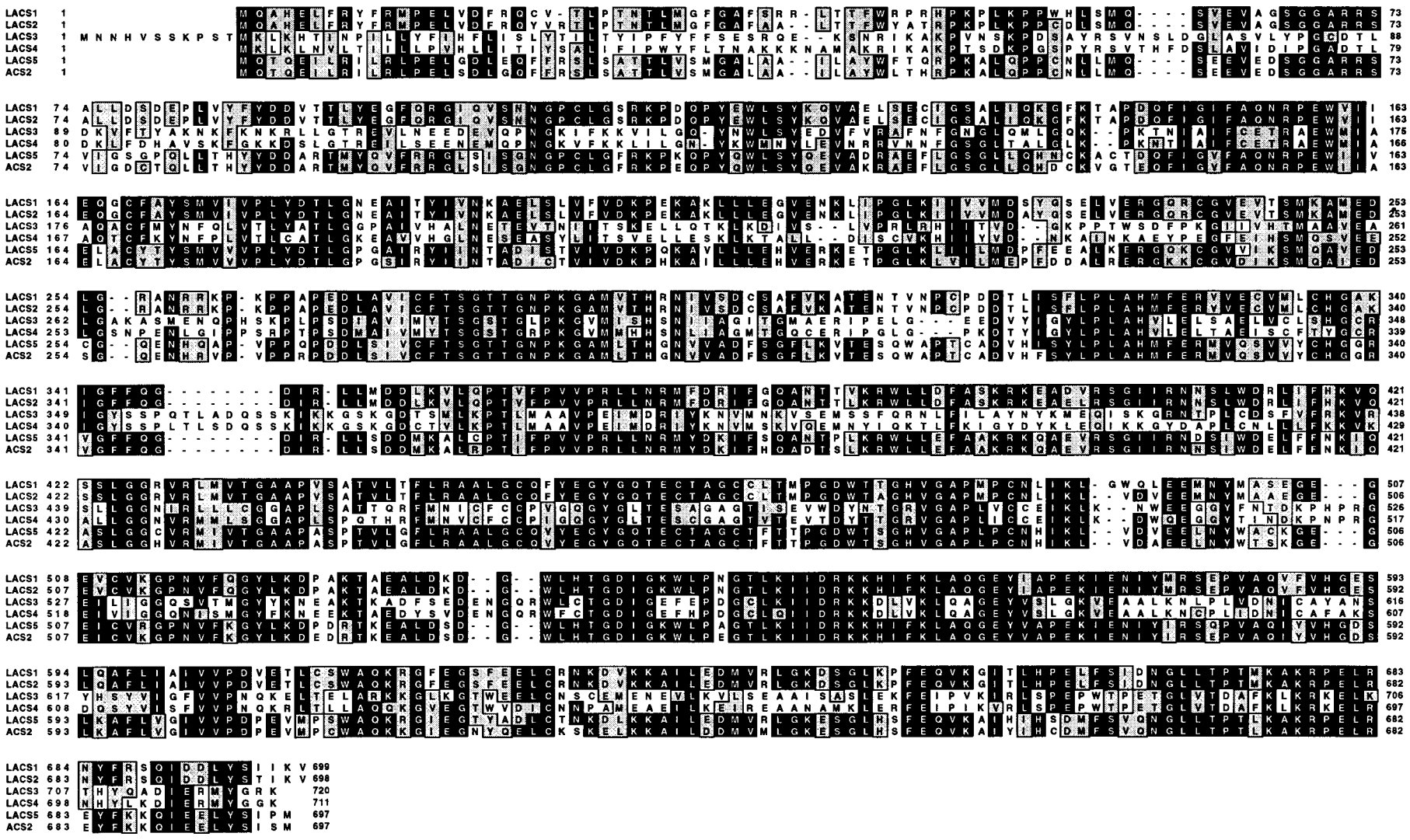


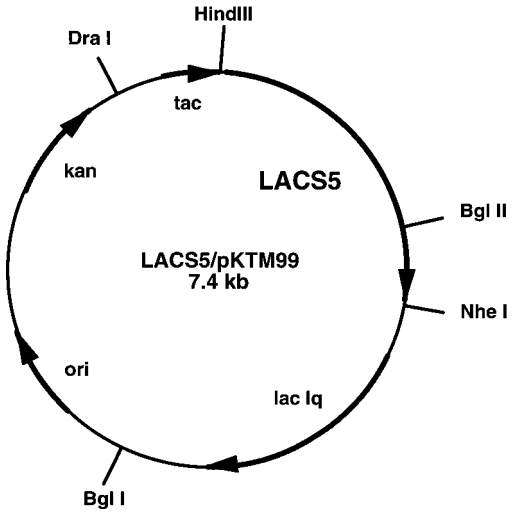
Figure 2 Amino acid sequence comparison of six acyl-CoA synthetase proteins

Human LACS5 is compared with human LACS1, LACS2, LACS3, LACS4 and rat ACS2. Identical amino acids are boxed in black. Similar amino acids are shown in grey. Non-identical residues are omitted. Dashes represent spaces introduced to optimize the alignment. Multiple alignments were done with the Clustal W 1.4 program (Mac Vector 6.5).

Table 1 Percentage amino acid sequence identities between human LACS5 and other reported acyl-CoA synthetases

Overall identities on human LACS1 to LACS5 and rat ACS1 to ACS5 are given as percentage amino acid identities. Individual pairwise alignments were conducted with Clustal W (1.4) multiple sequence alignment (MacVector 6.5).

	Amino acid identity (%)										
	LACS5	LACS4	LACS3	LACS2	LACS1	ACS5	ACS4	ACS3	ACS2	ACS1	Reference
LACS4	30	100									[9]
LACS3	28	62	100								[8]
LACS2	66	28	30	100							[7]
LACS1	65	28	30	96	100						[6]
ACS5	60	28	31	61	60	100					[22]
ACS4	30	97	67	30	30	29	100				[15]
ACS3	28	62	92	29	29	29	62	100			[14]
ACS2	91	28	29	64	63	57	28	29	100		[16]
ACS1	65	27	30	85	83	60	27	30	64	100	[13]



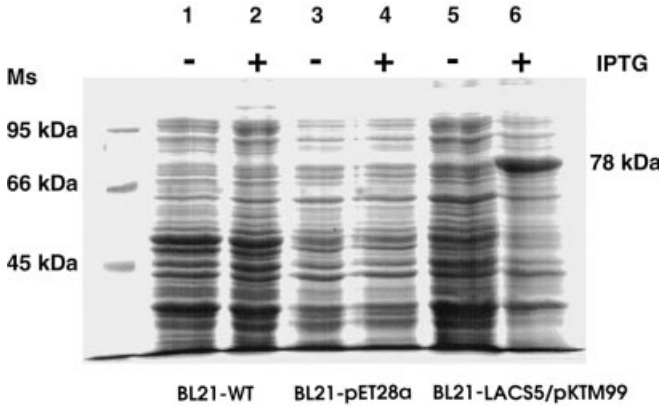


Figure 3 Overexpression of LACS5

Upper panel: schematic representation of plasmid LACS5/pKTM99, which overproduces LACS5 in *E. coli*. The solid arrow indicates the direction of the LACS5 gene transcription. The *tac* promoter, restriction sites and other genes are indicated. Lower panel: SDS/PAGE analysis of LACS5 in BL21 cells. The different BL21 cells were grown in the absence (−) or presence (+) of IPTG; the protein samples were denatured and separated by SDS/PAGE [10% (w/v) gel]. Results are given for wild-type BL21 cells (BL21-WT) in lanes 1 and 2, BL21 cells transfected with the vector BL21-pET28a in lanes 3 and 4, and BL21 cells containing the LACS5-expressing plasmid BL21-LACS5/pKTM99 in lanes 5 and 6. The positions of molecular mass markers are indicated at the left.

of LACS5 is compatible with a protein that is localized at a membrane/water interface. The actual membrane-binding sequence remains to be confirmed by the use of other, more direct, techniques on purified LACS5, reconstituted in bilayers of well-defined lipid composition.

A comparison of the sequences of LACS1 and LACS5 from K-562 cells and the other reported human acyl-CoA synthetases (LACS2–4) and rat brain ACS2 is given in Figure 2. The results indicate that the sequence identity of LACS5 with LACS1 and LACS2 is approx. 30% and with LACS 3 and LACS4 approx. 65%. Interestingly, it seemed that LACS5 from a human erythroid cell line had a high sequence identity with ACS2, a form of acyl-CoA synthetase found in rat brain (Figure 2). The extent of sequence similarities between the different acyl-CoA synthetases from human and rat origin is given in Table 1. Whereas LACS5 has high similarity to rat brain ACS2, the similarity is much less pronounced with any of the other human or rat forms, including the recently reported ACS5 [22]. Historically, the numbering of the (iso)forms in rat and human seems to have followed a similar numbering sequence, reflecting their similarity or tissue specificity as indicated in Table 1. However, our results indicate that the new LACS from K-562 cells has a much higher similarity to rat ACS2 than with the recently reported ACS5 [22] or the human LACS2 with rat ACS2. This suggests that it might be important to re-evaluate the nomenclature of these different forms of acyl-CoA synthetase from human and rat to avoid obvious confusion. On the basis of our results it would be sensible to give human LACS5 and rat ACS2 similar numerical designations. However, this cannot be accomplished without renaming the reported forms.

To show that the derived sequence of LACS5 encoded a functional protein, we constructed an expression plasmid LACS5/pKTM99 as indicated in Figure 3 (upper panel). The entire open reading frame of LACS5 cDNA was subcloned into the pET28a vector as described in the Materials and methods section and BL21 cells were transformed with the plasmid. Wild-type BL21 cells, BL21 cells transformed with the pET28a vector and cells containing the LACS5/pKTM99 plasmid were grown in the presence or absence of IPTG. Cell lysate was prepared and the protein composition and acyl-CoA activity were measured as shown in Figure 3 (lower panel) and Table 2. Both wild-type BL21 cells and BL21 cells transformed with the pET28a vector grown in the presence or absence of IPTG showed only a small amount of protein with an apparent molecular mass of 78 kDa (Figure 3, lower panel, lanes 1–4). Similarly, the cells transfected with LACS5/pKTM99, grown in the absence of IPTG, showed only a small amount of protein at this molecular mass (Figure 3, lower panel, lane 5). Induction of the transcription of the LACS5/pKTM99 plasmid resulted in a marked increase in a 78 kDa protein (Figure 3, lower panel, lane 6). These results indicate that cells transfected with the LACS5/pKTM99 plasmid containing LACS5 expressed a protein with a molecular mass close to that predicted by the amino acid sequence as given in Figure 1. Although these results clearly indicate that the overproduction of LACS5 can be accomplished in BL21 cells, it was important to show that this protein also acted as an acyl-CoA synthetase. The lysates were analysed on acyl-CoA synthetase activity with palmitic acid, oleic acid or arachidonic acid as substrate (Table 2). The results indicate the amount of oleoyl-

Table 2 Acyl-CoA activity in BL21 cell lysates

Cell lysates were tested for their ability to generate oleoyl-CoA, palmitoyl-CoA and arachidonoyl-CoA from oleic acid, palmitic acid and arachidonic acid as described in the Materials and methods section. The amount of acyl-CoA formed in 20 min from 10 nmol of fatty acid is given for wild-type BL21 cells (BL21-WT), BL21 cells transfected with the empty vector (BL21-EV) and BL21 cells containing the LACS5 plasmid (BL21-LACS5), grown in the presence (+) or absence (−) of IPTG. Results are means ± S.D.; $n = 3$. Abbreviation: n.d., not determined.

		Acyl-CoA formed (nmol/20 min per 10^8 cells)		
Lysate	IPTG	Oleoyl-CoA	Palmitoyl-CoA	Arachidonoyl-CoA
BL21-WT	−	0.20 ± 0.10	n.d.	n.d.
	+	0.30 ± 0.15	n.d.	n.d.
BL21-EV	−	0.25 ± 0.10	n.d.	n.d.
	+	0.23 ± 0.15	n.d.	n.d.
BL21-LACS5	−	0.46 ± 0.50	n.d.	n.d.
	+	5.1 ± 0.8	4.7 ± 0.8	2.3 ± 3.1

CoA formed in 20 min in the lysate of 10^8 cells. BL21 cells have a low level of endogenous oleoyl-CoA ligase activity and the introduction of the pET28a vector or the presence of IPTG did not significantly affect the ligase activity. The cells that contained the LACS5/pKTM99 plasmid exhibited only a small increase in ligase activity compared with the wild-type BL21 cells in the absence of IPTG. The induction with IPTG resulted in a marked increase in oleoyl-CoA ligase activity in these cells, in accord with the expression of the LACS5 protein as shown in Figure 3 (lower panel). Similarly, cells that expressed LACS5 showed a marked increase in their ability to form acyl-CoA with palmitate and arachidonate as substrates (Table 2). Taken together, these results show that LACS5 is expressed as an active enzyme in BL21 cells; similarly to erythrocyte ghosts, they can use palmitic, oleic or arachidonic acid as substrate. These fatty acyl groups are abundantly present in erythrocyte membrane phospholipids and are activated by erythrocyte membrane acyl-CoA synthetase [12]. Further detailed enzymic characterization of LACS5 in BL21 cells will render information of only limited value. The activity of LACS5 is likely to be affected by its membrane environment, as was recently shown for acyl-CoA synthetase activity from rat liver [24]; results on LACS5 in a poorly defined microbial lipid environment do not add to our knowledge of the function of this protein in erythrocytes. Most of the enzymology studies on acyl-CoA synthetases have been performed on either intact membranes containing possibly more than one acyl-CoA synthetase or purified proteins in poorly defined lipid environments, which makes comparison difficult. Although more specific enzyme kinetic studies are important, such studies need to be performed with these proteins reconstituted in a well-defined lipid environment.

To assess the presence of LACS5 mRNA in different erythroid precursors and human tissues, we performed a Northern blot analysis of total RNA. Figure 4(a) shows two major transcripts 2.9 and 6.3 kb in length. The LACS5 mRNA transcript of 2.9 kb is strongly expressed in K-562 cells, fetal blood derived from liver cells (which contains approx. 60–80 % nucleated erythrocytes), reticulocytes, bone marrow and cord blood. The transcripts are present, although to a much lower level, in 7-day-old and 14-day-old CD34-positive cells and are very low or absent in peripheral blood. The 6.3 kb mRNA transcript is strongly expressed in erythrocytes derived from fetal liver, and present at a lower level in bone marrow, cord blood, reticulocytes and K-562 cells. It is apparent from Figure 4(b) that LACS5 is also expressed in human brain as 9.4 and 2.9 kb transcripts. No information is available on the expression of rat ACS2 in rat erythroid cells, although on the basis of the sequence similarity it seems plausible that ACS2 would be the counterpart of the human erythroid acyl-CoA synthetase in rat erythrocytes. The 2.9 kb transcript is probably the closest to the translated sequence of LACS5 (2555 bp; Figure 1, upper panel). The 6.3 and 9.4 kb transcripts might include untranslated regions of mRNA. The difference in the relative amounts of the transcripts in erythroid tissues or the difference in length of the putative untranslated regions in erythroid tissues or brain might be the result of alternative mRNA splicing. Alternatively, similarities with other genes might underlie the presence of these transcripts.

In contrast with erythroid cells and brain, LACS5 mRNA transcripts are virtually absent from human heart, kidney, liver, lung, pancreas, spleen and skeletal muscle (Figure 4b). The other form of acyl-CoA synthetase, isolated from the K562 library, was very similar to human liver LACS1 [7]. To assess the presence of LACS1 mRNA in different erythroid precursors and human tissues we performed a Northern blot analysis of total RNA as described above for LACS5. In tissues other than erythroid, we found a similar expression of LACS1 mRNA to that was reported previously [7] (results not shown). Figure 4(c) shows the presence of a 3.6 kb LACS1 mRNA transcript in liver and erythroid cells. In contrast with the relatively low expression of LACS5 in early erythroid progenitors (7-day-old and 14-day-old CD34-positive cells), LACS1 showed a relatively high expression of a 3.6 kb transcript in these cells (Figure 4c). The 3.6 kb LACS1 transcript was present in K562 cells but was undetectable in reticulocytes, in contrast with the high expression of LACS5 mRNA (Figure 4a). These results suggest that the two proteins are transcribed differently during the development of the erythrocyte. The high expression in the reticulocytes suggested that LACS5 is present in the mature erythrocyte.

To test this hypothesis, we raised antibodies directed against LACS5 as described in the Materials and methods section and tested the cross-reactivity of rabbit sera towards the proteins in BL21 cells expressing LACS5, and erythrocyte ghosts by Western blotting (Figure 5). As expected, antibodies in the serum of rabbits before immunization with the LACS5 peptide reacted with different proteins in BL21 cells and in human erythrocyte ghosts (Figure 5, lanes 1 and 2). After immunization with a peptide designed on the basis of the sequence of LACS5, a protein band of approx. 78 kDa in BL21 cells as well as in erythrocyte ghosts was recognized by the rabbit antiserum (Figure 5, lanes 3 and 4).

Taken together, our results indicate that erythrocyte precursors expressed at least two forms of acyl-CoA synthetase: LACS1 and LACS5. On the basis of the mRNA present, LACS5 is expressed predominantly in erythrocyte precursors, in particular in reticulocytes, whereas LACS1 expression is found in most human tissues, but in contrast with LACS5 it is virtually absent from reticulocytes. Given the importance of long-chain acyl-CoA in lipid synthesis and β-oxidation, it is likely that all tissues and cells contain one or more (iso)forms of acyl-CoA synthetase for the generation of new lipid or the degradation of fatty acids. In blood cells, acyl-CoA synthetase activity has been reported in platelets [11,25] and erythrocytes [12]. The mature form of these cells in the peripheral blood is distinctly different from their precursors with respect to acyl-CoA utilization. In the earlier stages of erythrocyte development, lipid synthesis *de novo* and β-oxidation are important. However, lipid synthesis *de novo* and β-oxidation are absent from mature erythrocytes. The cell, which lacks internal organelles, generates acyl-CoA only for use in plasma membrane processes, including lipid remodelling and

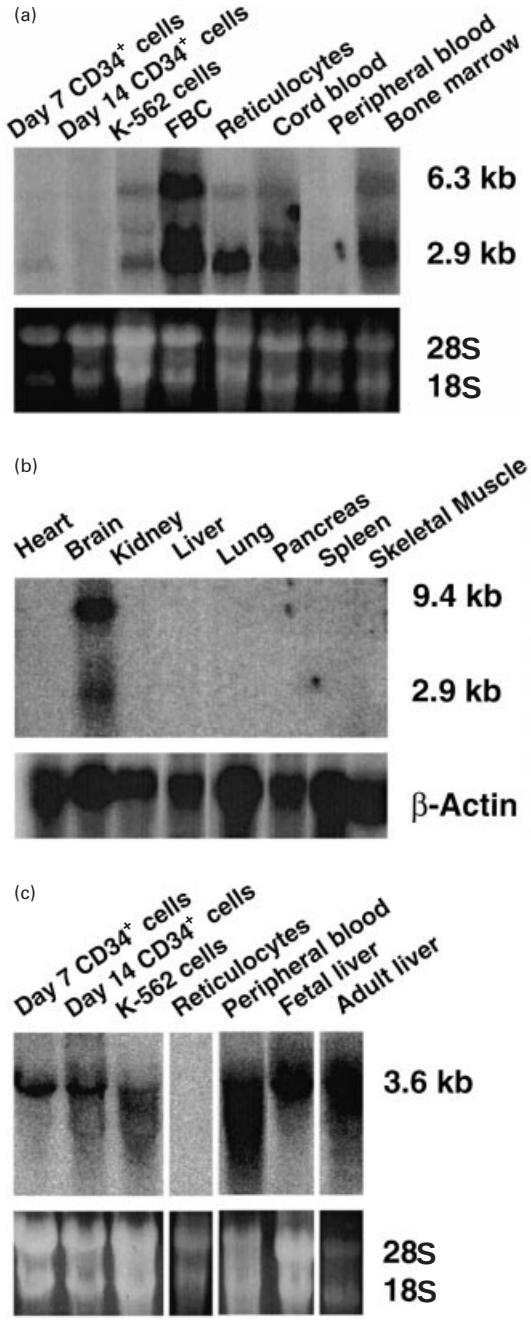


Figure 4 Northern blot analysis of the expression of LACS5 and LACS1 mRNA in various human tissues

(**a**) Total RNA (10–20 μg) extracted from a variety of erythrocyte precursors was separated, transferred on a Nytran membrane and probed with LACS5 cDNA as described in the Materials and methods section. The loading was verified by staining the gel with ethidium bromide (lower panel). FBC, fetal blood cells. (**b**) The use of a Human Territory Blot (Invitrogen) shows the expression of LACS5 mRNA in other human tissues. The lower panel shows the hybridization of the same membrane with a β-actin probe to verify RNA loading. (**c**) Total RNA (10–20 μg) extracted from a variety of erythrocyte precursors was separated, transferred on a Nytran membrane and probed with LACS1 cDNA as described in the Materials and methods section. The loading was verified by staining the gel with ethidium bromide (lower panel).

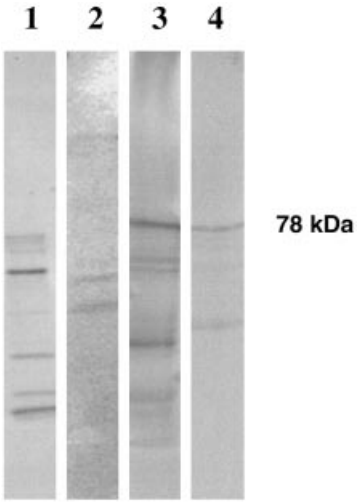


Figure 5 Western blot analysis of LACS5

Proteins of IPTG-induced BL21-LACS5 cells (lanes 1 and 3) and erythrocyte ghosts (lanes 2 and 4) were separated by SDS/PAGE, blotted on nitrocellulose membranes and identified by using antisera from rabbits before (lanes 1 and 2) and after immunization (lanes 3 and 4) with a peptide based on the LACS5 sequence as described in the the Materials and methods section.

protein acylation. Little is known about the structural differences between the reported acyl-CoA synthetases in relation to their subcellular localizations. However, given the very different roles of acyl-CoA synthesis in endoplasmic reticulum, in mitochondria and in plasma membranes, it might very well be that different forms of acyl-CoA synthetase are synthesized that are specific to these locations.

The presence of LACS1 in most human tissues tested, and in particular the high levels in human liver, suggest a role for this protein in lipid synthesis *de novo* or β-oxidation. LACS1 expression is found during the early stages of erythroid development, in which β-oxidation is present, and lipid synthesis *de novo* is important for the generation of (new) membrane material. In contrast, the expression of LACS1 is very low in reticulocytes and young erythrocytes, which are virtually devoid of *de novo* lipid synthesis or β-oxidation. In contrast, the mRNA signal for LACS5 is very high in reticulocytes, and lower in earlier stages of erythroid development. The high expression in the reticulocytes suggested that LACS5 is present in the mature erythrocyte membrane, which was confirmed by the cross-reactivity of rabbit sera towards the proteins in BL21 cells that express LACS5 and erythrocyte ghosts (Figure 5). The low transcription level of LACS5 in most tissues, including liver, suggests that this protein does not have a major role in lipid synthesis *de novo* or β-oxidation. However, the high expression of LACS5 mRNA in reticulocytes and its presence in erythrocyte ghosts suggest that LACS5 is the plasma membrane bound acyl-CoA synthetase activity found in mature erythrocytes [2–4,12]. Because processes of lipid and protein acylation are not unique to erythrocyte plasma membranes, it might very well be that this form of acyl-CoA synthetase is present in plasma membranes of other cells and tissues. The relatively low levels of LACS5 in other cells, or even its apparent absence, might be due to the low levels of this protein compared with those acyl-CoA synthetases involved in the intracellular generation of acyl-CoA.

In conclusion, we report a new human LACS, which we identify as LACS5, that is distinctly different from other human acyl-CoA synthetases. The protein uses long-chain fatty acyl groups as substrate, is highly expressed in erythrocyte precursors and might be the form that is important in the remodelling of plasma membrane lipids and proteins.

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REFERENCES

1 Lands, W. E. M. and Hart, P. (1965) J. Biol. Chem. **240**, 1905–1911
2 Mulder, E. and van Deenen, L. L. M. (1965) Biochim. Biophys. Acta **106**, 106–117
3 Renooij, W., van Golde, L. M. G., Zwaal, R. F. A., Roelofsen, B. and van Deenen, L. L. M. (1974) Biochim. Biophys. Acta **363**, 287–292
4 Das, A. K., Dasgupta, B., Bhattacharya, R. and Basu, J. (1997) J. Biol. Chem. **272**, 11021–11025
5 Arduini, A., Mancinelli, G., Radatti, G. L., Dottori, S., Molajoni, F. and Ramsay, R. R. (1992) J. Biol. Chem. **267**, 12673–12681
6 Ghosh, B., Barbosa, E. and Singh, I. (1995) Mol. Cell. Biochem. **151**, 77–81
7 Abe, T., Fujino, T., Fukuyama, R., Minoshima, S., Shimizu, N., Toh, H., Suzuki, H. and Yamamoto, T. (1992) J. Biochem. (Tokyo) **111**, 123–128
8 Minekura, H., Fujino, T., Kang, M.-J., Fujita, T., Endo, Y. and Yamamoto, T. T. (1997) Genomics **42**, 180–181
9 Cao, Y., Traer, E., Zimmerman, G. A., McIntyre, T. M. and Prescott, S. M. (1998) Genomics **49**, 327–330
10 Piccini, M., Vitelli, F., Bruttini, M., Pober, B. R., Jonsson, J. J., Villanova, M., Zollo, M., Borsani, G., Ballabio, A. and Renieri, A. (1998) Genomics **47**, 350–358
11 Wilson, D. and Majerus, P. (1982) J. Biol. Chem. **267**, 3510–3515
12 Davidson, B. C. and Cantrill, R. C. (1985) FEBS Lett. **193**, 69–74
13 Suzuki, H., Kawarabayasi, Y., Kondo, J., Abe, T., Nishikawa, K., Kimura, S., Hashomoto, T. and Yamamoto, T. (1990) J. Biol. Chem. **265**, 8681–8685
14 Fujino, T., Kang, M.-J., Suzuki, H., Iijima, H. and Yamamoto, T. (1996) J. Biol. Chem. **271**, 16748–16752
15 Kang, M.-H., Fujino, T., Sasano, H., Minekura, H., Yabuki, N., Nagura, H., Iijima, H. and Yamamoto, T. T. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 2880–2884
16 Fujino, T. and Yamamoto, T. (1992) J. Biochem. (Tokyo) **111**, 197–203
17 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18 Malhotra, K., Luehrsen, K. R., Costello, L.L, Raich, T. J., Sim, K., Foltz, L., Davidson, S., Xu, H., Chen, A., Yamanishi, D. T. et al. (1999) Nucleic Acids Res. **27**, 839–847
19 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) J. Mol. Biol. **215**, 403–410
20 Beaumelle, B. D. and Vial, H. J. (1988) Biochim. Biophys. Acta **958**, 1–9
21 Sheibani, N. and Frazier, W. A. (1998) Biotechnique **25**, 28–32
22 Oikawa, E., Iijima, H., Suzuki, T., Sasano, H., Sato, H., Kamataki, A., Nagura, H., Kang, M.-J., Fujino, T., Suzuki, H. and Yamamoto, T. T. (1998) J. Biochem. (Tokyo) **124**, 679–685
23 Anderson, L. C. and Nilsson, K. G. (1979) Int. J. Cancer **23**, 143–147
24 Marra, C. A. and de Alaniz, M. J. T. (1999) Lipids **34**, 343–354
25 Bakken, A. and Holmsen, H. (1991) Biochem. J. **274**, 145–152

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