The mouse *Nudt7* gene encodes a peroxisomal nudix hydrolase specific for coenzyme A and its derivatives

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A mouse homologue of the *Saccharomyces cerevisiae* Pcd1p coenzyme A diphosphatase, NUDT7α, has been expressed as a thioredoxin fusion protein in *Escherichia coli*. NUDT7α is also a CoA diphosphatase of the nudix hydrolase family, and hydrolyses CoA, CoA esters and oxidized CoA with similar efficiences, yielding 3′,5′-ADP and the corresponding 4′-phosphopantetheine derivative as products. K_m and k_{cat} values with CoA were 240 μM and 3.8 s^{-1}. Activity was optimal at pH 8.0 with 5 mM Mg^{2+} or Mn^{2+} ions, while fluoride was inhibitory with an IC_{50} value of 20 μM. Expression of the *Nudt7* gene was highest in liver, intermediate in lung and kidney, and lowest in brain and heart, producing a 1.5 kb transcript. A similar pattern of expression was found for the human orthologue, *NUDT7*. An enzymically inactive splice variant, NUDT7β, which lacks 20 amino acids downstream of the nudix motif, was also found to be expressed in mouse tissues. Transfection of HeLa cells with a vector expressing the *Nudt7*α gene fused to the C-terminus of red fluorescent protein showed that NUDT7α, like Pcd1p, was a peroxisomal enzyme. The function of the NUDT7 enzyme may be the elimination of oxidized CoA from peroxisomes, or the regulation of CoA and acyl-CoA levels in this organelle in response to metabolic demand.

Key words: fatty acid oxidation, peroxisomes, pyrophosphatase.

INTRODUCTION

The nudix hydrolases are a widespread family of enzymes that function to eliminate potentially toxic nucleotide metabolites from the cell, and to regulate the concentrations and availability of many different nucleotide substrates, cofactors and signalling molecules [1,2]. All family members possess the nudix box sequence signature motif (nudix or NUDT motif) GX_5EX_5(UA)XRE(UA)XEEXGU (where U is a hydrophobic amino acid). The majority are nucleotide diphosphatases (pyrophosphatases) that hydrolyse the diphosphate linkage in compounds of general structure NDP-X, yielding NMP and P_i-X, where X can be another nucleotide or non-nucleotide moiety, e.g. NDP-sugars, NADH, dinucleoside polyphosphates and nucleoside 5′-triphosphates [1,3]. Exceptions to this are the diphosphoinositol polyphosphate phosphorylase subfamily, which can hydrolyse the non-nucleotide diphosphoinositol polyphosphates in addition to diadenosine polyphosphates [4,5], and the *Escherichia coli* GDP-mannose mannosyl hydrolase, which, unlike all other known nudix hydrolases, catalyses a nucleophilic substitution at a carbon atom to yield GDP and mannose as products [6].

Recently, we added CoA and its derivatives to the list of nudix hydrolase substrates [7]. These compounds are hydrolysed by Pcd1p, a peroxisomal nudix hydrolase from *Saccharomyces cerevisiae*, which is one of five nudix family members found in this organism [7]. In view of its preference for oxidized CoA as substrate, we suggested that the function of Pcd1p may be to eliminate oxidized CoA from the highly oxidizing environment of the peroxisomes during fatty acid β-oxidation [7]. In addition to the nudix motif, Pcd1p has the upstream signature LLTXR(SA)$X_3RX_3GX_3$FPGG, designated UPF0035 in the PROSITE database (see http://ca.expasy.org/cgi-bin/nicedoc.pl?PDOC00995, and Figure 1 below). This motif is also present in related nudix hydrolase sequences in prokaryotes and other eukaryotes, including mammalian cells. To confirm that this motif is responsible for determining substrate specificity for CoA and its derivatives, and to initiate an investigation of the importance of CoA-metabolite turnover in mammalian peroxisomes, we have cloned, expressed and characterized two closely related Pcd1p homologues from the mouse. One of these, NUDT7α, is a peroxisomal CoA diphosphatase, and the other, NUDT7β, appears to be a minor, inactive splice variant.

EXPERIMENTAL

Materials

All nucleotides and CoA derivatives were from Sigma. [^{32}P]UTP (800 Ci/mmol) was from ICN. Calf intestinal alkaline phosphatase, yeast inorganic pyrophosphatase, *Eco*RI, *Nco*I, *Xho*1 and the pET32b(+) expression vector were from Novagen. *Pfu* DNA polymerase was from Stratagene and *Taq* polymerase was from Amersham Pharmacia Biotech. IMAGE (integrated molecular analysis of genomes and their expression consortium) clones 1891167 and 1922917 were obtained from the Human

Abbreviations used: DsRed, *Discosoma* red fluorescent protein; EST, expressed sequence tag; GFP, green fluorescent protein, IMAGE, integrated molecular analysis of genomes and their expression consortium; IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani broth; Ni-NTA, Ni^{2+}-nitrilotriacetate; ORF, open reading frame; PTS, peroxisomal targeting signal; RT-PCR, reverse-transcriptase PCR; SKL, C-terminal tripeptide PTS1; Trx, thioredoxin.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession number AF338424.

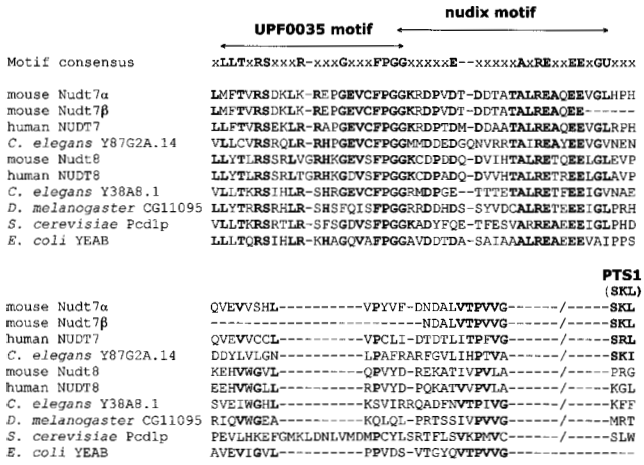


Figure 1 Partial sequence alignment of NUDT7α and NUDT7β with related sequences

Sections of the NUDT7α and NUDT7β sequences encompassing the PROSITE UPF0035 and nudix motifs, the 20 amino acid sequence missing in NUDT7β, and the C-terminal tripeptide were aligned with a selected number of related sequences retrieved by a BLAST search using the CLUSTAL X algorithm. Amino acid residues common to at least half of the sequences are shown in **bold**. Accession numbers for the sequences depicted are: mouse NUDT7α, AF338424; mouse NUDT7β, AA244635; human NUDT7, AA227330; *Caenorhabditis elegans* Y87G2A.14, CAB54476; mouse NUDT8, AI854862; human NUDT8, AI743601; *C. elegans* Y38A8.1, AAA98017; *Drosophila melanogaster* CG11095, AAF48325; *S. cerevisiae* Pcd1p, Z73323; *E. coli* YEAB, P43337.

Genome Mapping Project (Hinxton Hall, Cambridge, U.K.). The peptide fusion vector pGFP-SKL, where the mammalian green fluorescent protein (GFP) is fused to the C-terminal tripeptide peroxisomal targeting signal 1 (PTS1), SKL, was a generous gift from P. Van Veldhoven (Department of Molecular and Cell Biology, Catholic University, Leuven, Belgium).

Cloning of NUDT7α and NUDT7β cDNAs

The IMAGE clone 1891167 contains an insert that includes the full-length open reading frame (ORF) of the putative 236-amino-acid NUDT7α protein cloned between the *Dra*III sites of the vector pME18S-FL3. The sequence of the insert was confirmed and the ORF was then amplified by PCR using the 29-bp forward (AGTACTGCCATGGTGATGTCGCGACCTTG) and reverse (TTTTGGAATTCAGGGTCTTCACAACTTGC) primers. These primers were synthesized to provide an *Nco*I restriction site at the start of the amplified ORF and an *Eco*RI site at the end. After amplification with *Pfu* DNA polymerase, the DNA was recovered by phenol/chloroform extraction and digested with *Nco*I and *Eco*RI. The digest was gel purified and the required restriction fragment ligated between the *Nco* I and *Eco*RI sites of the pET32b(+) thioredoxin (Trx)-fusion vector. The resulting construct, pET-NUDT7α, generated a fusion of NUDT7α downstream of the cleavable 109-amino-acid Trx-fusion protein, and His-tagged and S-tagged sequences. This plasmid was used to transform *E. coli* XL1-Blue cells for propagation. The IMAGE clone 1922917 contains an insert that potentially encodes a 216-amino-acid protein lacking a 20-amino-acid sequence to the C-terminal side of the NUDT7α nudix motif, which we have termed NUDT7β. This was also cloned as described above to give the construct pET-NUDT7β.

Protein expression and purification

E. coli strain BL21(DE3) was transformed with pET-NUDT7α. A single colony was picked from a Luria–Bertani broth (LB) agar plate containing 60 μg/ml ampicillin and inoculated into 10 ml of LB medium containing 60 μg/ml ampicillin. After overnight growth at 37 °C, the cells were transferred to 1 l of LB medium containing 60 μg/ml ampicillin and grown to a D_{600} of 0.8 at 37 °C. Isopropyl β-D-thiogalactoside (IPTG) was added to a concentration of 1 mM, and the cells were incubated for 2 h 30 min. The induced cells were harvested, washed and re-suspended in 25 ml of breakage buffer (50 mM Tris/HCl, pH 8.0, and 0.1 M NaCl). The cell suspension was sonicated, and the resulting lysate cleared by centrifugation at 15000 ***g*** at 4 °C for 10 min. The supernatant was recovered and applied to a 15 mm × 50 mm column of Ni^{2+}-nitrilotriacetate (Ni-NTA)–agarose (Sigma) equilibrated with 20 mM Tris/acetate, pH 7.0, 0.3 M NaCl and 10 mM 2-mercaptoethanol, at a flow rate of 0.5 ml/min. After eluting the unbound proteins, a linear gradient of 0–50 mM histidine in equilibration buffer was applied at a flow rate of 1 ml/min, and fractions of 1 ml were collected and analysed by SDS/PAGE. Those containing pure Trx–NUDT7α fusion protein were collected, dialysed overnight against 50 mM Tris/acetate, pH 7.0, 100 mM NaCl and 1 mM dithiothreitol, and concentrated by ultrafiltration. NUDT7β protein was expressed from pET-NUDT7β and purified in an identical manner.

Enzyme assays and product identification

Substrates were screened by measuring the P_i released in a coupled assay as described previously [8,9]. Non-specific alkaline phosphodiesterase (nucleotide pyrophosphatase) activity was assayed using thymidine-5′-monophospho-*p*-nitrophenyl ester as substrate [10]. Kinetic parameters and reaction products generated from CoA and its derivatives were determined by high performance anion-exchange chromatography. Reaction mixtures containing 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, substrate in the range 0.1–1.0 mM and 0.5 μg of Trx–NUDT7α fusion protein were incubated at 37 °C for up to 20 min in a volume of 200 μl. Samples (100 μl) of the reaction mix were analysed by HPLC as described previously [7].

Preparation of C-terminus of *Discosoma* red fluorescent protein (DsRed)–NUDT7α fusion construct and subcellular localization

A construct, expressing a fusion of NUDT7α to the C-terminus of human codon-optimized DsRed, was constructed by PCR amplification of the NUDT7α-coding region from the IMAGE clone 1891167 using the 28- and 29-bp forward and reverse primers, AGAGGAAGTACTCGAGGGCTGATGTCGC and TTTTGGAATTCAGGGTCTTCACAACTTGC respectively, to provide an *Xho*I restriction site at the start of the amplified region and an *Eco*RI site at the end. After amplification with *Pfu* DNA polymerase, the DNA was recovered by phenol/chloroform extraction and digested with *Xho*I and *Eco*RI. The digested and gel-purified PCR product was ligated between the *Xho*I and *Eco*RI sites of the pDsRed1-C1 vector (Clontech) to give pDsRed1-C1–NUDT7α. For subcellular localization, HeLa cells were transfected with pDsRed1-C1–NUDT7α using the FuGENE™ 6 transfection reagent (Roche Diagnostics) as described by the manufacturer's instructions. Briefly, cells were grown in 35 mm glass-bottomed dishes (MatTek, Ashland, MA, U.S.A.) to 50% confluence in Dulbecco's modified Eagle's medium, supplemented with 10% foetal-calf serum. FuGENE™

6 (3 μl) was diluted in 100 μl serum-free medium, incubated for 5 min at room temperature and added dropwise to 1 μg of construct DNA. The mixture was then incubated for 15 min at room temperature and added dropwise to the cell monolayer in a total volume of 2 ml. After an overnight incubation at 37 °C, the subcellular localization of the expressed protein was determined by using a Zeiss LSM510 confocal microscope with a 100×1.4 NA objective. Cells were transfected with pGFP-SKL in an identical manner.

Northern and dot blots

A ^{32}P-labelled DNA probe was generated from a full-length NUDT7α-PCR product using a random-labelling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. A Northern blot containing total RNA from various mouse tissues (10 μg/lane) was prepared by standard procedures [9], and probed with the DNA probe (10^6 c.p.m./ml) at 64 °C overnight. The blot was washed for 2×15 min with 0.15 M NaCl and 0.015 M sodium citrate containing 0.5 % SDS at 64 °C, and the bound ^{32}P-labelled probe was detected using a PhosphorImager (Bio-Rad). A Human RNA Master Blot™ dot blot (Clontech), containing normalized loadings of poly(A)$^+$ RNA from 50 different human tissues, was probed and processed as described above.

Reverse-transcriptase PCR (RT-PCR)

A full-length first-strand cDNA corresponding to the NUDT7α transcript was reverse transcribed from total RNA from 9 different mouse tissues (Ambion, Austin, TX, U.S.A.) using a first strand cDNA synthesis kit (MBI Fermentas, Munich, Germany) according to the manufacturer's instructions. Briefly, 5 μg of total RNA, 20 pmol of 29-bp reverse primer (TTTTGGAATTCAGGGTCTTCACAACTTGC), 10 mM dNTPs, 20 units of ribonuclease inhibitor and 40 units of reverse transcriptase were mixed, and then incubated at 37 °C for 1 h. The reaction was stopped by heating at 70 °C for 10 min. First strand cDNA (5 μl) was used in subsequent standard PCR reactions as described above, except that *Taq* DNA polymerase was used instead of *Pfu* DNA polymerase. The PCR products were separated on a 1 % agarose gel.

Other methods

Protein concentrations were estimated by the Coomassie-Blue-binding method [11].

RESULTS

Cloning, expression and purification of NUDT7α and NUDT7β Trx-fusion proteins

A BLAST search of the GenBank® mouse expressed sequence tag (EST) database with the *S. cerevisiae* Pcd1p sequence yielded a large number of mouse clones with a high degree of similarity to Pcd1p which, when assembled, revealed two distinct but closely related sequences. The first was a 1052 bp sequence, with a 708 bp ORF, potentially encoding a 236-amino-acid 26.9 kDa protein with both a nudix motif and the PROSITE UPF0035 motif, which we have previously suggested may indicate a hydrolytic specificity for CoA and its derivatives [7]. This sequence was termed *Nudt7*α in accordance with the mouse and human gene nomenclature guidelines for the nudix protein family (see http://www.gene.ucl.ac.uk/users/hester/npym.html). The second sequence, *Nudt7*β, was identical with that of *Nudt7*α except for the loss of 60 nt encoding a 20-amino-acid stretch which includes the C-terminal end of the nudix motif (Figure 1). That the predicted sequence of *Nudt7*α represents the full-length protein is shown by the presence of multiple stop codons both upstream and downstream of the ORF, and by the highly favourable sequence context of the proposed initiator codon (underlined), AAAATGG [12].

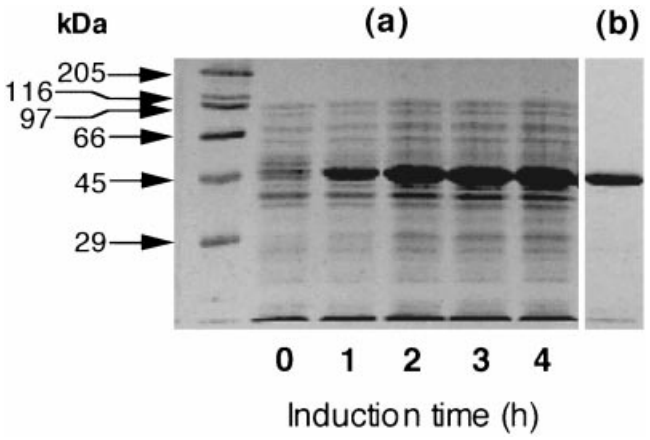


Figure 2 Expression and purification of Trx–NUDT7α fusion protein

(**a**) *E. coli* BL-21(DE3) transformed with pET-NUDT7α was induced with 1 mM IPTG for up to 4 h. Aliquots were taken at hourly intervals, boiled in sample buffer, analysed by SDS/PAGE on a 13 % gel and stained with Coomassie Blue. (**b**) Trx–NUDt7α fusion protein was purified on a Ni-NTA–agarose column as described in the Experimental section, and a sample of the pooled peak was analysed by SDS/PAGE as above. Protein standards are: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa.

The NUDT7α cDNA sequence was PCR amplified from the IMAGE clone 1891167, which was predicted to contain the full-length cDNA. For expression, the PCR fragment was inserted into the pET32b(+) vector, and the recombinant plasmid, pET-NUDT7α, was then used to transform *E. coli* BL-21(DE3) cells to generate a His-tagged Trx-fusion protein with the predicted mass of 43.2 kDa. After IPTG induction, cell samples were analysed by SDS/PAGE, which showed that the soluble fraction contained a major IPTG-inducible protein band migrating with an apparent molecular mass of 45 kDa. This was presumed to be the desired product (Figure 2a). The expressed Trx–NUDT7α fusion protein was then purified in a single step, to apparent homogeneity, on a Ni-NTA–agarose column (Figure 2b). The NUDT7β variant in IMAGE clone 1922917 was cloned and expressed in the same way. In this case, most of the expressed protein appeared in inclusion bodies. However, Trx–NUDT7β was successfully purified from the soluble fraction as described above, yielding a protein of apparent molecular mass 44 kDa (results not shown). N-terminal sequencing confirmed the identity of both the expressed proteins.

Properties of the enzymic activity

As predicted, Trx-NUDT7α showed substantial hydrolytic activity with CoA and CoA derivatives (Table 1), further substantiating the role of the UPF0035 motif in determining specificity towards this group of compounds. No activity was found with NDP-sugars, CDP-alcohols, (deoxy)nucleoside 5′-triphosphates, nucleoside 5′-di- or mono-phosphates, di-adenosine polyphosphates, NAD$^+$, NADH, NADP$^+$, NADPH or thymidine-5′-monophospho-*p*-nitrophenyl ester, even when the

Table 1 Kinetic parameters of Trx-NUDT7α for the hydrolysis of CoA and CoA-derivatives

Kinetic parameters were obtained under standard HPLC assay conditions as described in the Experimental section. The values quoted are the means of duplicate determinations. Individual values were within 10% of the mean.

Substrate	K_m (mM)	V_{max} (μmol/min per mg of protein)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
CoA	0.240	5.3	3.8	16.0
Acetyl-CoA	0.430	7.6	5.5	12.6
CoA–S–S–CoA	0.235	3.0	2.1	9.0
Succinyl-CoA	0.330	4.1	3.0	9.0
3′-dephospho-CoA	0.480	2.2	1.6	3.3

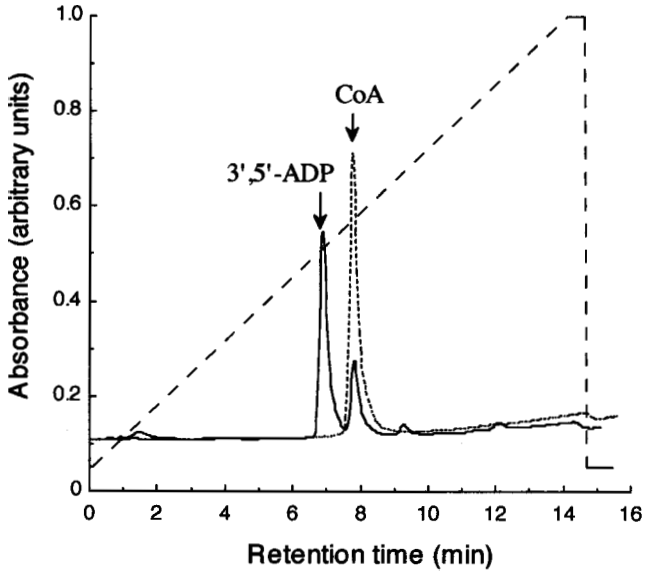


Figure 3 Identification of reaction products from the hydrolysis of CoA by NUDT7α

Assays containing 0.3 mM CoA were incubated for 30 min with 0.5 μg of Trx–NUDT7α fusion protein, then applied to a 1 ml Resource-Q anion-exchange column as described previously [7]. Broken line, without enzyme; continuous line, with enzyme. The gradient and elution positions of standards are indicated.

enzyme concentration was increased 10-fold. HPLC analysis of the products of CoA hydrolysis showed that the enzyme was a CoA diphosphatase, cleaving the diphosphate linkage in CoA to give 3′,5′-ADP and 4′-phosphopantetheine (Figure 3). The enzyme displayed optimal activity at pH 8.0 with either 5 mM Mg^{2+} or Mn^{2+} ions. In common with all other nudix hydrolases tested, fluoride was inhibitory, with an IC_{50} value of approximately 20 μM using 1 mM CoA as the substrate (results not shown).

The K_m and k_{cat} values for CoA and its derivatives ranged from 235–480 μM and 1.6–5.5 s^{-1} respectively (Table 1). For CoA, the K_m and k_{cat} values of 240 μM and 3.8 s^{-1} are very close to those determined for yeast Pcd1p (280 μM and 2.6 s^{-1} [7]). In contrast, the K_m of NUDT7α for oxidized CoA (235 μM) was about 10-fold higher than that of Pcd1p (24 μM); so, whereas Pcd1p has a 13-fold higher k_{cat}/K_m ratio for oxidized CoA compared with CoA, the corresponding value for NUDT7α is about 0.6. Thus NUDT7α does not appear to favour oxidized CoA as a substrate.

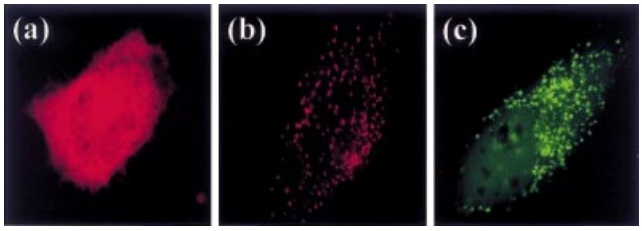


Figure 4 Subcellular localization of NUDT7α protein

HeLa cells were transfected with (**a**) pDsRed1-C1 DNA expressing DsRed, (**b**) pDsRed1-C1–NUDT7α DNA expressing NUDT7α fused to the C-terminus of DsRed, and (**c**) pGFP-SKL DNA, expressing a peroxisomally-targeted GFP. Fluorescence was revealed using a Zeiss LSM510 confocal microscope with a 100 × 1.4 NA objective.

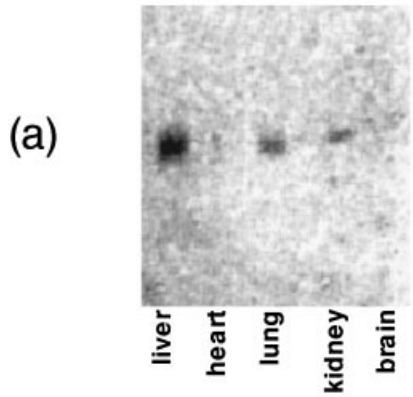
When NUDT7β was examined for enzyme activity, none was detected, even though the UPF0035 motif and most of the nudix motif are intact. Presumably loss of the 20-amino-acid stretch in this variant causes a structural alteration sufficient to abolish enzyme activity. Thus NUDT7β appears to be a minor, non-functional variant.

Subcellular localization

S. cerevisiae Pcd1p is targeted to peroxisomes, the sole site of fatty acid β-oxidation in yeast, by an N-terminal PTS2 [7]. Examination of the NUDT7α sequence shows it to have a typical C-terminal tripeptide PTS1, SKL (Figure 1). In order to determine if this SKL sequence locates NUDT7α to peroxisomes, HeLa cells were transfected with the construct pDsRed1-C1–NUDT7α, which expresses the NUDT7α sequence fused to the C-terminus of human codon-optimized DsRed, and then examined by confocal microscopy. Cells transfected with the pDsRed1-C1 vector alone showed a diffuse cytoplasmic fluorescence with no clear subcellular localization (Figure 4a), while cells transfected with pDsRed1-C1–NUDT7α showed a clear punctate fluorescence pattern which is characteristic of peroxisomes (Figure 4b). A similar pattern of discrete fluorescent spots was observed in cells transfected with a construct expressing an authentic, peroxisomal targeted GFP–SKL (Figure 4c). These results indicate that NUDT7α is a peroxisomal protein, and is directed towards these organelles by the C-terminal PTS1 sequence, SKL.

Tissue distribution of NUDT7α and NUDT7β mRNA expression

Northern- and dot-blot analyses were performed to determine the size and tissue distribution of NUDT7 transcripts. No attempt was made to distinguish between the NUDT7α and NUDT7β mRNAs. A transcript of approximately 1.5 kb, assumed to be predominantly NUDT7α, was detected in all the mouse tissues represented on the Northern blot (Figure 5a). Expression appeared highest in liver, intermediate in lung and kidney, and lowest in brain and heart. The human *Nudt7*α homologue, *NUDT7*, displays 66% sequence identity with *Nudt7*α gene, and almost certainly represents the orthologous sequence. Therefore, a NUDT7α cDNA was also used to probe a dot blot of 50 human



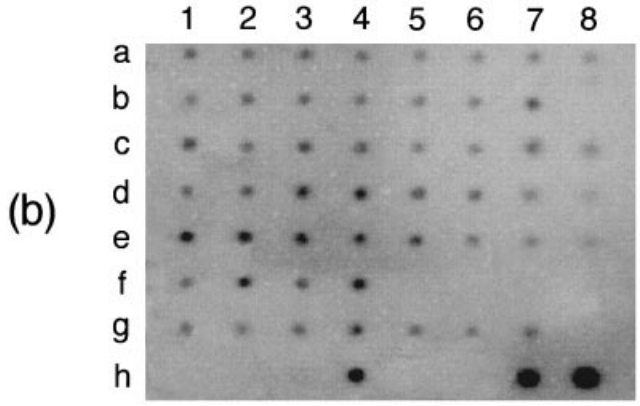


Figure 5 Expression of mouse NUDT7 and human NUDT7 RNA in different tissues

(**a**) Total mouse RNA prepared from 5 different tissues was subjected to Northern-blot analysis (10 μg/lane) and probed with a ^{32}P-labelled NUDT7α cDNA as described in the Experimental section. (**b**) A commercial RNA dot blot containing normalized loadings of 89–514 ng of each poly(A)$^+$ RNA per dot from 50 different human tissues and 100 ng each of six control RNAs and DNAs (500 ng for dot h8) was probed as above. Dot identification: a1, whole brain; a2, amygdala; a3, caudate nucleus; a4, cerebellum; a5, cerebral cortex; a6, frontal lobe; a7, hippocampus; a8, medulla oblongata; b1, occipital lobe; b2, putamen; b3, substantia nigra; b4, temporal lobe; b5, thalamus; b6, nucleus accumbeus; b7, spinal cord; c1, heart; c2, aorta; c3, skeletal muscle; c4, colon; c5, bladder; c6, uterus; c7, prostate; c8, stomach; d1, testis; d2, ovary; d3, pancreas; d4, pituitary gland; d5, adrenal gland; d6, thyroid gland; d7, salivary gland; d8, mammary gland; e1, kidney; e2, liver; e3, small intestine; e4, spleen; e5, thymus; e6, peripheral leucocytes; e7, lymph node; e8, bone marrow; f1, appendix; f2, lung; f3, trachea; f4, placenta; g1, foetal brain; g2, foetal heart; g3, foetal kidney; g4, foetal liver; g5, foetal spleen; g6, foetal thymus; g7, foetal lung; h1, yeast total RNA; h2, yeast tRNA; h3, *E. coli* rRNA; h4, *E. coli* DNA; h5, poly r(A); h6, human C_0t 1 DNA; h7, human DNA; h8, human DNA.

tissue mRNAs that had been normalized with respect to eight different housekeeping genes (Figure 5b). The results confirmed those of the mouse Northern blots, showing high to moderate expression in liver (e2 and g4), lung (f2) and kidney (e1), as well as in pancreas (d3), pituitary (d4), small intestine (e3), spleen (e4) and placenta (f4). Expression in brain tissues was generally low (a1–b7), while expression in the heart (c1) appeared higher than was evident from the Northern blot. In general, the pattern of expression was similar to that found for the human NUDT5 ADP-sugar diphosphatase [9].

The relative expression of NUDT7α and NUDT7β mRNA in different mouse tissues was examined by RT-PCR as described in the Experimental section. A major band corresponding to the

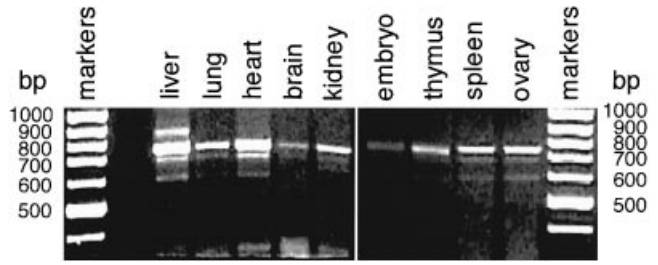


Figure 6 Detection of NUDT7α and NUDT7β RNA expression by RT-PCR

Total RNA from nine different mouse tissues was reverse transcribed, PCR amplified and separated on a 1% agarose gel as described in the Experimental section. The two panels show two separate experiments involving different RNA samples.

expected NUDT7α 744-bp PCR product was apparent in all tissues (Figure 6). This product was sensitive to cleavage by *Ban*1, which has a unique recognition site within the 60-nucleotide sequence missing from NUDT7β transcript, and was confirmed as NUDT7α by sequencing. In addition, all the tissues displayed a band just below NUDT7α. To confirm that this was the expected 684-bp NUDT7β product, the products of a PCR reaction were digested with *Ban*1 to remove the bulk of the NUDT7α, run on a gel, and the minor band was isolated, re-amplified and sequenced. The confirmed NUDT7β transcript was estimated to comprise 5–10% of the total. The identities of the other minor bands arising in certain tissues were not investigated further. NUDT7β is almost certainly a minor splice variant arising from the removal of the 60 nt as an additional intron. This intron has a canonical GT-donor-splice site and branch-point sequence, but a non-canonical AT-acceptor site. The donor and acceptor sites are in a suitable sequence context for splicing, but are presumably used inefficiently as a splice-site pair. This alternative splicing may not occur in humans due to lack of a suitable branch-point sequence, and there are presently no examples of a NUDT7β-type sequence in the human EST database. However, several human ESTs identical with NUDT7, but lacking the complete nudix motif and all or nearly all of the UPF0035 motif, can be found in the GenBank® Database (e.g. accession numbers AA315404 and AV662116). Whether mouse NUDT7β and these human variants are translated *in vivo* and have any physiological function remains to be determined.

DISCUSSION

Our finding that the mouse *Nudt7* gene encodes a nudix hydrolase specific for CoA and its derivatives confirms the PROSITE UPF0035 motif as a likely determinant of hydrolytic specificity towards this nucleotide. Recently, a report was published indicating that the UPF0035-containing nudix hydrolase from *Deinococcus radiodurans* was also a CoA diphosphatase [13]. Thus the UPF0035 motif can be added to those features that determine the substrate specificities and structure–function relationships within the various nudix hydrolase subfamilies [3]. A detailed understanding of these features is essential in order to help predict the functions of the rapidly increasing number of nudix hydrolases revealed by genome sequencing. Given that Lys occurs at position 9 in 23 of the 26 database sequences containing this motif (compared with the six cited in PROSITE), and Val or Ile occurs in 21 of these 26 sequences, we would suggest that the consensus for UPF0035 should be refined to

LLTXR[SA]X_2LRX_3GX[VI]XFPGG. Site-directed mutagenesis of this motif should confirm its importance in substrate binding and specificity.

The fact that the Pcd1p CoA diphosphatase from *S. cerevisiae* has a 10-fold lower K_m for oxidized CoA relative to CoA led us to suggest that its function may be the elimination of potentially toxic or non-functional oxidized CoA generated within the oxidizing environment of the peroxisomes [7]. Confirmation of this awaits the results of experiments with a *PCD1* deletion mutant. The results in this study with mouse NUDT7α show that, although it is also peroxisomal, it does not appear to have a preference for oxidized CoA *in vitro* (Table 1). Nevertheless, oxidized CoA could still be the favoured substrate *in vivo*. The total CoA pool (CoA plus acyl-CoA) in rat liver peroxisomes has been estimated at around 300 μM [14], similar to the K_m values of Nudt7α for these compounds; however, the bulk of this may be protein-bound and not free [15]. If upon generation, oxidized CoA were to be released free into the matrix as a non-functional oxidation product, then it would become preferentially available as a substrate for NUDT7α. Since the β-oxidation of fatty acids in yeast is confined to peroxisomes, the exclusive peroxisomal location of yeast Pcd1p (the only yeast nudix hydrolase active towards CoA and its derivatives), it suggests that this subfamily of nudix hydrolases functions either to remove oxidized CoA or to regulate or maintain the supply of CoA and/or its derivatives for fatty acid oxidation, rather than for any other metabolic pathway involving CoA. The high level of NUDT7 transcript expression in liver and the low level of expression in brain is consistent with the relative involvement of these organs in fatty acid oxidation.

Animal cells have three principal pools of CoA: the cytosolic pool provides CoA mainly for fatty acid biosynthesis, the mitochondrial pool is required for the oxidation of fatty acids (in contrast with yeast), pyruvate and some amino acids, and the peroxisomal pool functions in the initial oxidation of very long-chain fatty acids prior to their continued and complete oxidation in the mitochondria. Thus it might be expected that the animal mitochondrial compartment should also possess CoA-diphosphatase activity. Although NUDT7α appears to be exclusively peroxisomal, animal cells (at least in mammals and *Caenorhabditis elegans*) express a second nudix hydrolase with the UPF0035 motif that appears to dictate specificity for CoA derivatives (Figure 1). Like NUDT7α and the human orthologue NUDT7, the *C. elegans* Y87G2A14 protein possesses a likely C-terminal PTS1 (Figure 1). On the other hand, the mouse NUDT8, human NUDT8, *C. elegans* Y38A8.1 and *Drosophila melanogaster* CG11095 sequences possess neither a PTS1 nor a PTS2. Analysis of the *D. melanogaster* CG11095 sequence with the MITOPROT algorithm led to a 97% prediction of a mitochondrial location [16], while analysis on the PSORT Web server (http://psort.nibb.ac.jp/) indicated a 44% probability of this protein being mitochondrial. Hence if this location and the enzyme activity of these proteins are experimentally confirmed, the need for the regulation or cleansing of the CoA pool for fatty acid oxidation within this organelle would be satisfied. If, as suggested, a major aspect of this function is the elimination of oxidized CoA, the reducing environment of the cytosol might preclude the need for such an enzyme activity in this compartment.

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