Mitochondrial and peroxisomal targeting of 2-methylacyl-CoA racemase in humans

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Abstract 2-Methylacyl-CoA racemase is an auxiliary enzyme required for the peroxisomal β-oxidative breakdown of (2*R*)-pristanic acid and the (25*R*)-isomer of C_{27} bile acid intermediates. The enzyme activity is found not only in peroxisomes but also is present in mitochondria of human liver and fibroblasts. The C terminus of the human racemase, a protein of 382 amino acids with a molecular mass of 43,304 daltons as deduced from its cloned cDNA, consists of KASL. Hitherto this sequence has not been recognized as a peroxisomal targeting signal (PTS1). From the in vitro interaction between recombinant racemase and recombinant human PTS1 receptor (Pex5p), and the peroxisomal localization of green fluorescent protein (GFP) fused to the N terminus of full-length racemase or its last six amino acids in tranfected Chinese hamster ovary (CHO) cells, we concluded that ASL is a new PTS1 variant. To be recognized by Pex5p, however, the preceding lysine residue is critical. As shown in another series of transfection experiments with GFP fused to the C terminus of the full-length racemase or racemase with deletions of the N terminus, mitochondrial targeting information is localized between amino acids 22 and 85. Hence, our data show that a single transcript gives rise to a racemase protein containing two topogenic signals, explaining the dual cellular localization of the activity. —Amery, L., M. Fransen, K. De Nys, G. P. Mannaerts, and P. P. Van Veldhoven. **Mitochondrial and peroxisomal targeting of 2-methylacyl-CoA racemase in humans. *J. Lipid Res.* 2000.** 41: **1752–1759.**

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In mammals, peroxisomes are responsible for the β-oxidative degradation of isoprenoid-derived carboxylates such as pristanic acid and the C_{27} bile acid intermediates, di- and trihydroxycoprostanic acid. The four reactions involved are catalyzed by a specific set of enzymes, different from those responsible for the β-oxidation of straight-chain fatty acids (1). In humans, these enzymes are named branched-chain acyl-CoA oxidase, the D-specific multifunctional protein 2, and SCPx-thiolase (1). Pristanic acid, formed via α-oxidation of racemic phytanic acid, is racemic at position 2 (2, 3), whereas in vivo-synthesized C_{27} bile intermediates possess a (25*R*)-methyl group (4). The oxidases acting on branched compounds recognize only (2*S*)-pristanoyl-CoA (5) and (25*S*)-trihydroxycoprostanoyl-CoA (5, 6). Hence, in addition to the enzymes mentioned above, extra enzymes seem to be necessary for the degradation of (2*R*)-pristanic acid and (25*R*)-trihydroxycoprostanic acid. Only one enzyme, discovered by Schmitz, Fingerhut, and Conzelmann (7) and named 2-methylacyl-CoA racemase, which interconverts the α-methyl groups of the CoA-esters by proton abstraction, appears to be required.

In humans, like in rats, racemase activity is present both in peroxisomes and in mitochondria (8, 9). The mitochondrial enzyme is likely to assist in the breakdown of pristanic acid metabolites, shortened in the peroxisomes and shuttled to the mitochondria for further degradation (10). Also these metabolites contain a (2*R*)-methyl branch (2) and the involved mitochondrial dehydrogenases act only on the (2*S*)-methyl isomers (11, 12). Racemases, purified from rat liver mitochondrial fractions (7) or from total human liver homogenates (8), are monomers of approximately 45–47 kDa. By immunohybridization, the rat and mouse racemase cDNAs were cloned (13). The encoded proteins, with a molecular mass of 39,679 and 39,558 Da, respectively, appear to be identical to 2-arylpropionyl-CoA epimerase (14), an enzyme discovered during studies of the active form of Ibuprofen, a nonsteroidal anti-inflammatory drug. The rat racemase protein, as well as its mouse counterpart, ends in KANL (13, 14), a sequence known to be a peroxisome-targeting signal (peroxisomal targeting signal 1, PTS1) for human catalase (15). However, no evidence of a mitochondrial targeting signal, normally present at the N terminus of mitochondrial matrix proteins, was seen in the cDNA-deduced rat and mouse racemase sequences and Northern analysis revealed only a single mRNA species (13, 14). Given our in-

Abbreviations: EST, expressed sequence tag; GST, glutathione *S*-transferase; GFP, green fluorescent protein; IMAGE, Integrated Molecular Analysis of Genomes and their Expression; PCR, polymerase chain reaction; PTS1, peroxisomal targeting signal 1; RACE, rapid amplification of cDNA ends; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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TABLE 1. List of primer sequences[a]

Name	Sequence	Remarks
HsRac1r	5′-AAGGGATGGCTGGGGTGTTT	F
HsRac2r	5′-CCACTCTGCCTTCGTCTTCTTTGC	F
HsRac3r	5′-GCCATGAATTCCCCATCTGC	F
HsRac4s	5′-GGAGATCTTGGGAAGCGCCATGGCACTGC	*Bgl*II
HsRac5r	5′-GGGGTACCGTCGACTTGAGCCGTGGGCCTG	*Sal*I
HsRac6s	5′-GCACTGGGTACCGCCATGGCACTGCAGG	*Kpn*I
HsRac7r	5′-GGGCCTAGATCTTAGAGACTAGCTTTTACC	*Bgl*II
HsRac8r	5′-GGGCCTGGAAGATCTAGACTAGCTTTTACC	*Bgl*II
HsRac9s	5′-GGCCCGTTCGGTACCATGGTCCTGGCTG	*Kpn*I
HsRac10s	5′-GCCGCGGTACCATGGAGAAACTCCAGCTG	*Kpn*I
HsRac11r	5′-GCTGGAGTAGATCTATGACACCGCGGCGG	*Bgl*II

[a] Restriction sites introduced in the primers are underlined; the symbols s and r stand for sense and reverse primers, respectively; F indicates the presence of a 5′-fluorescein label.

terests in peroxisomal β-oxidation and in identifying branched-chain acyl-CoA oxidase deficiencies, we realized that phenotypically such oxidase and racemase deficiencies might be similar. Hence, we tried to obtain more information about the human racemase and its targeting to peroxisomes and/or mitochondria (16).[2]

EXPERIMENTAL PROCEDURES

Cloning and expression of recombinant racemase

To obtain the human racemase cDNA sequence, we used the rat racemase/epimerase amino acid sequence (13, 14) as a query to search (17) the expressed sequence tag (EST) database for corresponding human ESTs. Plasmids, isolated from selected IMAGE [Integrated Molecular Analysis of Genomes and their Expression; ref. (18)] clones (AA453310, clone 788180; AA122152, clone 548722; AA085247, clone 547229) (HGMP-RC, Hinxton, Cambridge, UK), were sequenced (cycle sequencing kit; Pharmacia, Uppsala, Sweden) with fluorescently labeled vector primers and subsequently with gene-specific primers (HsRac1r/F, HsRac2r/F, and HsRac3r/F; see **Table 1** for primer sequences). Clones 547229 and 548722, however, were shown to be mouse derived. To obtain the 5′ end of the human racemase cDNA, a bidirectionally cloned λgt11 human liver cDNA library [random- and oligo (dT)-primed] (Clontech, Palo Alto, CA) was subjected to 5′ rapid amplification of cDNA ends (5′-RACE) protocols using the λgt11 3′ or 5′ phage-specific primers and HsRac1r/F, followed by a second polymerase chain reaction (PCR) with a nested gene-specific primer, HsRac2r/F. The resulting amplicons were restricted with *Eco*RI, subcloned in pBluescript SK(+), and sequenced with the corresponding vector primers.

To perform interaction studies with the PTS1 receptor Pex5p, a 600-bp *Eco*RI fragment of clone 788180 was inserted into *Eco*RI-treated pGEX-4T-1 (Amersham Pharmacia, Piscataway, NJ), creating a vector encoding glutathione *S*-transferase (GST) fused to the last 157 amino acids of human racemase. This ligation mixture was used to transform *Escherichia coli* DH5α cells and clones containing the insert in the correct orientation were selected. The expression of the 40-kDa GST-(Δ1–288) HsRacemase fusion protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h and subsequently the fusion protein was purified on glutathione-Sepharose 4B according to the manufacturer instructions.

[2] Some parts of this article have been presented in preliminary form (16).

An amplicon encompassing the full-length racemase open reading frame was generated by PCR, using human liver cDNA (19) in combination with the racemase-specific primers HsRac4s and HsRac5r. The 1.16-kb specific PCR product was gel purified, digested with *Bgl*II and *Sal*I, and subcloned into the *Bam*HI/*Sal*I-digested two-hybrid system bait vector pGBT9 (Clontech) and the *Bam*HI/*Sal*I-digested pQE32 expression vector (Qiagen, Valencia, CA). These constructs, pMF282 and pMF323, encode $Gal4_{bd}$-HsRacemase and hexahistidine $(His)_6$-HsRacemase, respectively. To generate pMF244, a construct encoding GST-HsRacemase, we transferred the 1.16-kb *Eco*RI/*Sal*I fragment of pMF282, obtained by a partial digest, to pGEX-4T-1 digested with *Eco*RI and *Sal*I. Racemase activities were measured in lysates of *E. coli* Top10F′ expressing the $(His)_6$-HsRacemase or GST-HsRacemase as described previously (9).

Targeting studies

In vitro interactions of the recombinant racemase with recombinant biotinylated human Pex5p fusion protein were analyzed in microtiter plate and overlay assays as described previously (20, 21). For the analysis of in vivo interactions, two 24-mers, 5′-GTA CAAGGTAAAAGCTAGTCTCTA and 5′-GGCCTAGAGACTAGC TTTTACCTT, were allowed to hybridize and ligated into *Bsr*GI/*Not*I-restricted EGFP-N1 vector (Clontech). In the resulting plasmid, pLA34, the codons for L and Y of green fluorescent protein (GFP) are restored and the last GFP codon, encoding K, is replaced by a sequence encoding KVKASL. Another GFP construct, pLA39, encoding GFP-KVGASL, was prepared in a similar way using 5′-GTACAAGGTAGGTGCTAGTCTCTA and 5′-GGC CTAGAGACTAGCACCTACCTT as oligomers. Vectors containing the full-length racemase, fused either upstream (pLA63) or downstream (pLA72) of the GFP-coding region, were also constructed. Therefore, the full-length racemase was generated by PCR using human liver cDNA (19) and the racemase-specific primers HsRac6s and HsRac7r or HsRac8r, respectively. The specific PCR products were gel purified, digested with *Kpn*I/*Bgl*II, and subcloned into the *Kpn*I/*Bam*HI-digested pEGFP-C1 (pLA63) or pEGFP-N1 vector (pLA72), respectively. Other racemase-GFP constructs were made in a similar way, by subcloning the *Kpn*I/*Bgl*II-restricted respective PCR products into the *Kpn*I/*Bam*HI-digested pEGFP-N1 vector: pLA73 (amino acids 22 to end), using the primers HsRac9s and HsRac8r; pLA74 (amino acids 85 to end), using the primers HsRac10s and HsRac8r; pLA75 (amino acids 22 to 84), using the primers HsRac9s and HsRac11r; and pLA76 (amino acids 1 to 84), using the primers HsRac9s and HsRac11r.

Localization of GFP-fusion proteins in transfected Chinese hamster ovary cells was analyzed by indirect immunofluores-

cence microscopy as described previously (22). Colocalization studies were performed with 2 μM Mito-tracker (Molecular Probes, Eugene, OR) or immunostaining for Pex14p.

Generation of antisera

To raise a polyclonal antiserum against human racemase, the 40-kDa GST-(Δ1–288) HsRacemase, bound to the glutathione-Sepharose 4B, was first cleaved with thrombin and the released 18-kDa racemase fragment was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose, and, after dimethylsulfoxide dissolution (23), emulsified with an equal amount of Freund's complete adjuvant (priming dose; 70 μg of protein) or Freund's incomplete adjuvant (successive boosting doses; 60 μg of protein) and injected into a chicken. Pex14p and GST antisera were obtained by immunizing rabbits with $(His)_6$-tagged HsPex14p and GST, respectively. The first protein was expressed in *E. coli* Top10F′ cells transformed with plasmid pMF44 (24) and purified on nickel-nitrilotriacetic acid resin (Qiagen) according to the manufacturer-described procedure for denatured proteins. The 58-kDa $(His)_6$-HsPex14p was further purified via SDS-PAGE and transferred onto nitrocellulose. Strips containing 50 μg of protein were dissolved by adding dimethyl sulfoxide (23), emulsified with an equal amount of Freund's (in)complete adjuvant, and injected subcutaneously into a rabbit. GST was expressed in *E. coli* Top10F′ cells, transformed with pGEX-4T-1, and purified by glutathione-Sepharose 4B chromatography. The glutathione eluates were directly emulsified with Freund (in)complete adjuvant and injected subcutaneously (300 μg/dose).

RESULTS AND DISCUSSION

On the basis of the rat racemase amino acid sequences (13, 14), ESTs of the corresponding human protein were retrieved from the databases. The cDNA of the human 2-methylacyl-CoA racemase,[3] composed from overlapping sequences present in the IMAGE (18) EST clone 788810 and 5′-RACE products, obtained from a λgt11 library, contained an open reading frame of 1,149 bases encoding a protein of 42,304 Da (**Fig. 1**). Although 5′ information, including a possible start codon, was present in one EST (H19271; clone 171859),[4] we relied on 5′-RACE techniques to verify the presence/absence of differentially spliced mRNAs that could give rise to racemases with a different N terminus. Differential splicing might be a possible explanation for the dual localization of racemase activity in peroxisomes and mitochondria as observed in liver of rats and humans and in human fibroblasts (8, 9). All RACE products obtained, however, were derived from the same sequence and could be aligned to the sequence of EST H19271. Despite attempts with different constructs, we did not succeed in expressing human racemase in an active form in bacteria.

Amino acid similarities of the human racemase to mouse and rat racemase were both 87% (**Fig. 2**). Because of the identity with a sequence-tagged site (G21632; WI-

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-111                                                    ggattgggagggcttcttgca
 -61 ggctgctgggctggggctaagggctgctcagtttccttcagcggggcactgggaagcgcc
   1 atggcactgcagggcatctcggtcgtggagctgtccggcctggccccgggccgtttctgt
     M  A  L  Q  G  I  S  V  V  E  L  S  G  L  A  P  G  R  F  C      20
  61 gctatggtcctggctgacttcggggcgcgtgtggtacgcgtggaccggccccggctcccgc
     A  M  V  L  A  D  F  G  A  R  V  V  R  V  D  R  P  G  S  R      40
 121 tacgacgtgagccgcttgggccggggcaagcgctcgctagtgctggacctgaagcagccg
     Y  D  V  S  R  L  G  R  G  K  R  S  L  V  L  D  L  K  Q  P      60
 181 cggggagccgccgtgctgcggcgtctgtgcaagcggtcggatgtgctgctggagcccttc
     R  G  A  A  V  L  R  R  L  C  K  R  S  D  V  L  L  E  P  F      80
 241 cgccgcggtgtcatggagaaactccagctgggcccagagattctgcagcgggaaaatcca
     R  R  G  V  M  E  K  L  Q  L  G  P  E  I  L  Q  R  E  N  P     100
 301 aggcttatttatgccaggctgagtggatttggccagtcaggaagcttctgccggttagct
     R  L  I  Y  A  R  L  S  G  F  G  Q  S  G  S  F  C  R  L  A     120
 361 ggccacgatatcaactatttggctttgtcaggtgttctctcaaaaattggcagaagtggt
     G  H  D  I  N  Y  L  A  L  S  G  V  L  S  K  I  G  R  S  G     140
 421 gagaatccgtatgccccgctgaatctcctggctgactttgctggtggtggccttatgtgt
     E  N  P  Y  A  P  L  N  L  L  A  D  F  A  G  G  G  L  M  C     160
 481 gcactgggcattataatggctctttttgaccgcacacgcactgacaagggtcaggtcatt
     A  L  G  I  I  M  A  L  F  D  R  T  R  T  D  K  G  Q  V  I     180
 541 gatgcaaatatggtggaaggaacagcatatttaagttcttttctgtggaaaactcagaaa
     D  A  N  M  V  E  G  T  A  Y  L  S  S  F  L  W  K  T  Q  K     200
 601 tcgagtctgtgggaagcacctcgaggacagaacatgttggatggtggagcacctttctat
     S  S  L  W  E  A  P  R  G  Q  N  M  L  D  G  G  A  P  F  Y     220
 661 acgacttacaggacagcagatggggaattcatggctgttggagcaatagaaccccagttc
     T  T  Y  R  T  A  D  G  E  F  M  A  V  G  A  I  E  P  Q  F     240
 721 tacgagctgctgatcaaaggacttggactaaagtctgatgaacttcccaatcagatgagc
     Y  E  L  L  I  K  G  L  G  L  K  S  D  E  L  P  N  Q  M  S     260
 781 acggatgattggccagaaatgaagaagaagtttgcagatgtatttgcaaagaagacgaag
     T  D  D  W  P  E  M  K  K  K  F  A  D  V  F  A  K  K  T  K     280
 841 gcagagtggtgtcaaatctttgacggcacagatgcctgtgtgactccggttctgacttttt
     A  E  W  C  Q  I  F  D  G  T  D  A  C  V  T  P  V  L  T  F     300
 901 gaggaggttgttcatcatgatcacaacaaggaacggggctcgtttatcaccagtgaggag
     E  E  V  V  H  H  D  H  N  K  E  R  G  S  F  I  T  S  E  E     320
 961 caggacgtgagcccccgccttgcacctctgctgttaaacaccccagccatcccttcttcc
     Q  D  V  S  P  R  L  A  P  L  L  L  N  T  P  A  I  P  S  S     340
1021 aaaggggatcctttcataggagaacacactgaggagatacttgaagaatttggattcagc
     K  G  D  P  F  I  G  E  H  T  E  E  I  L  E  E  F  G  F  S     360
1081 cgagaagagatttatcagcttaactcagataaaatcattgaaagtaataaggtaaaagct
     R  E  E  I  Y  Q  L  N  S  D  K  I  I  E  S  N  K  V  K  A     380
1141 agtctctaacttccaggcccacggctcaagtgaatttgaatactgcatttacagtgtaga
     S  L  *                                                        382
1201 gtaacacataacattgtatgcatggaaacatggaggaacagtattacagtgtcctaccac
1261 tctaatcaagaaaagaattacagactctgattctacagtgatgattgaattctaaaaatg
1321 gttatcattagggcttttgatttataaaacttgggtacttatactaaattatggtagtt
1381 attctgccttccagtttgcttgatatatttgttgatattaagattcttgacttatatttt
1441 gaatgggttctagtgaaaaaggaatgatatattcttgaagacatcgatatacatttattt
1501 acactcttgattctacaatgtagaaaatgaggaaatgccacaaattgtatggtgataaaa
1561 gtcacgtgaaacagagtgattggttgcatccaggccttttgtcttggtgttcatgatctc
1621 cctctaagcacattccaaactttagcaacagttatcacacttttgtaatttgcaaagaaaa
1681 gtttcacctgtattgaatcagaatgccttcaactgaaaaaaacatatccaaaataatgag
1741 gaaatgtgttggctcactacgtagagtccagagggacagtcagttttagggttgcctgta
1801 tccagtaactcggggcctgtttccccgtgggtctctgggctgtcagctttcctttctcca
1861 tgtgtttgatttctcctcaggctggtagcaagttctggatcttatacccaacacacagca
1941 acatccagaaataaagatctcaggacccccaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 1. Nucleotide and deduced amino acid sequence of human 2-methylacyl-CoA racemase. The composed cDNA, numbered from the start codon and 2,111 bases long, is derived from overlapping sequences present in the IMAGE Consortium cDNA clone 788180 and different amplicons obtained by PCR of a λgt11 human liver cDNA library. Different methionine codons are present in frame near the 5′ end, whereby the first two fit to a partial Kozak rule (36). The first was used as start codon. A polyadenylation site (underlined) is found upstream of the poly(A) tail. In the full-length racemase clones obtained by PCR of a laboratory-made cDNA library, the gtg codon for Val-9 was replaced by atg, creating another methionine codon with a partial fit to the Kozak sequence. This base substitution probably reflects polymorphism.

[3] The cDNA sequence was deposited in the EMBL databank (AJ130733). During the course of our investigations, a human 2-methylacyl-CoA racemase sequence was deposited in the GenBank (AF047020, g2896147); this sequence was different from the one we had obtained, and was apparently a mixture of mouse and human sequences. Later it was replaced by a corrected version (g4204096) that, except for 10 nucleotides in the coding region resulting in 7-amino acid substitutions, was identical to our cDNA. Presumably, some of these differences represent polymorphisms.

[4] The cloning of the human 2-methylacyl-CoA racemase by RT-PCR and based on the 5′ information present in the EST H19271 sequence, was described by Ferdinandusse, S., S. Denis, P. T. Clayton, A. Graham, J. E. Rees, J. T. Allen, B. N. McLean, A. Y. Brown, P. Vreken, H. R. Waterham, and R. J. A. Wanders. 2000. Mutations in the gene encoding peroxisomal alpha-methylacyl-CoA racemase cause adult-onset sensory motor neuropathy. *Nat. Genet.* **24:** 188–191.

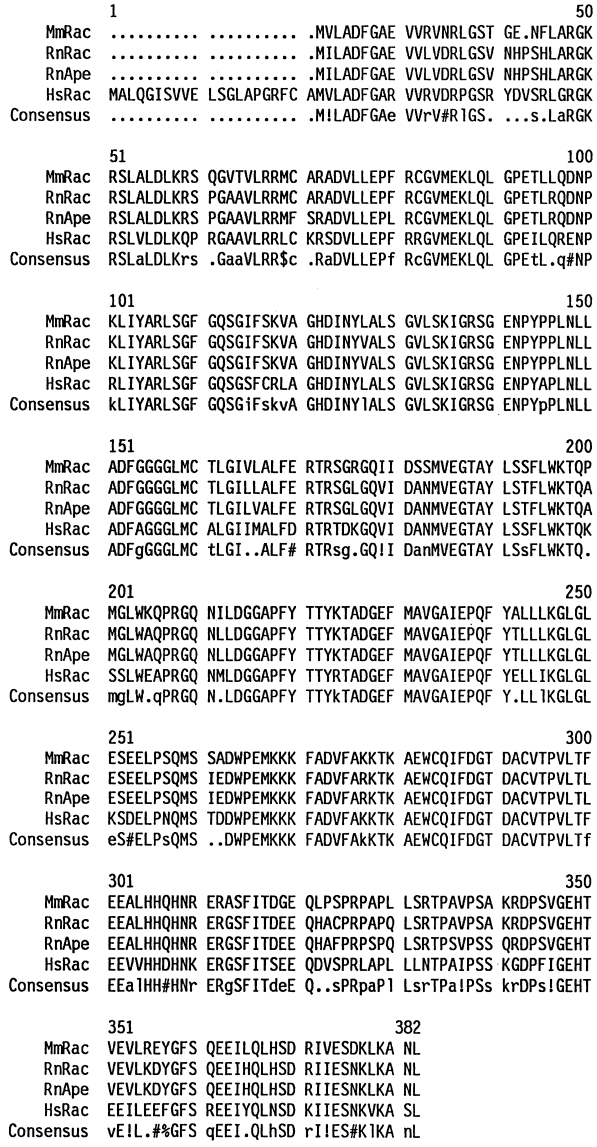

Fig. 2. Comparison of human 2-methylacyl-CoA racemase with mouse and rat racemases. The amino acid sequence deduced from the obtained human racemase cDNA (HsRac) was aligned (37) to the racemase from rat (RnRac) (13) and mouse (MmRac) (13) and aryl-propionyl-CoA epimerase from rat (RnApe) (14). Upper and lower case letters in the consensus line indicate, respectively, highly and weakly conserved residues. A dot in the consensus line represents a position with no conserved residues, while ! stands for I or V, $ for L or M, % for F or Y, and # for NDQE.

16117), human racemase could be localized to chromosome 5p11-13. According to the EST information, racemase is expressed in most human tissues (uterus, kidney, brain, colon, prostate, lung, lymph node, connective tissue, pancreas; UniGene Hs.128749). This is in agreement with the enzyme activity measurements in rat tissues (9). In the rat the highest mRNA levels are seen in liver (13, 14), but transcripts are also present in kidney, heart, and brain (14). Immunoblotting of rat tissues revealed clear signals only in liver and kidney (**Fig. 3**). Although racemase activity in human liver is severalfold lower than in rat liver (8, 9), the immunosignals obtained in these tissues were of a similar intensity (Fig. 3). This might be due to a better recognition of the human antigen by the antiserum used. Human skin fibroblasts contained a low amount of racemase protein (data not shown).

Most peroxisomal matrix proteins possess a C-terminal targeting signal, called PTS1, that interacts with the PTS1-receptor Pex5p. PTS1 was first defined as the tripeptide SKL, but in mammals many other variants are also active. Currently, the consensus has been broadened to **S**/A/C/K/N-**K**/R/H/Q/N-**L**/I (22, 25, 26). In addition, residues upstream of the tripeptide modulate the binding to Pex5p (15, 22, 25). In many cases, a positively charged and often critical residue is found at the fourth last position of mammalian PTS1-containing proteins (15, 25). Whereas the rat and mouse enzymes end in KANL (13, 14), the C terminus of human racemase is KASL. In contrast to the tetrapeptide KANL, a sequence known to target human catalase to peroxisomes (15), KASL has not been reported to be present in peroxisomal proteins or to be a functional PTS1 in mammals. Therefore, interactions with the PTS1 receptor were studied. As shown in **Fig. 4**, the C-terminal third of human racemase was recognized by human Pex5p and the racemase was displaced by a peptide ending in SKL. Pex5p bound also to a GST-full-length racemase fusion protein (data not shown). In addition, in cells transfected with constructs encoding a chimeric GFP ending in KVKASL, the last six amino acids of human racemase, a fluorescent peroxisomal pattern was seen. However, GFP-KVGASL remained cytosolic (**Fig. 5**), pointing toward the importance of the positive charge of the fourth last residue.

Compared with the mouse and rat protein sequences, human racemase has an extended amino terminus and its mature size, based on immunoblotting, is indeed larger (43 kDa for the human vs. 41 kDa for the rat protein; Fig. 3). Given the presence of various conserved arginines downstream of this extension, the N-terminal sequence might have a mitochondrial targeting function (27, 28). Database searches against known protein motifs and analysis by specific programs (MitoProt II; 27), however, failed to reveal such function. Hence, the intracellular fate of different GFP-racemase fusions was analyzed (Fig. 5). When GFP was situated N terminally of the full-length racemase, a peroxisomal staining was seen, in agreement with the localization of GFP-KVKASL. A C-terminally tagged construct, however, displayed a mitochondrial distribution. Because the 5′ end of the human cDNA contained more than one putative start codon, the region responsible for the mitochondrial targeting was delineated in more detail. When the second methionine (amino acid 22) was used as start codon, mitochondrial staining was still observed. However, fusion proteins starting at the third methionine (amino acid 85) remained cytosolic (Fig. 5). When only the region corresponding to amino acids 1 to 84 was fused to GFP, mitochondria were stained but also some cytosolic staining was seen (data not shown). Hence, the topogenic information is located between amino acids 22 and 84, a region that is well con-

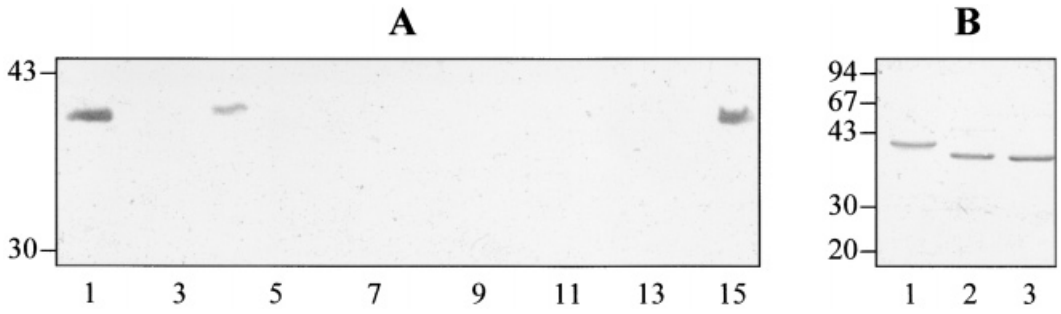

Fig. 3. Immunoblot analysis of mammalian tissues. Proteins present in homogenates of different rat tissues, prepared as decribed in ref. 9, and of human liver were separated by SDS-PAGE, blotted to nitrocellulose, and immunostained with chicken anti-racemase. (A) Lanes 1 to 15: liver, cerebrum, cerebellum, kidney, spleen, testis, lung, small intestinal mucosa, heart, pancreas, muscle, Harderian gland, thymus, and liver (50 μg of each; 10–20% gradient gels). (B) Lanes 1 to 3: human liver, rat liver of control animals, and rat liver of clofibrate-treated animals (20 μg of each; 10% gels). Migration of markers (size in kDa) is shown on the left. In the Harderian gland of rat, a tissue in which rather high racemase activities were reported (9), no immunosignals were seen. Treatment of the rats with clofibrate did not result in induction of the racemase.

served between rodent proteins and is likely to fulfill a similar targeting function.

Thus, our data indicate that in humans 2-methylacyl-CoA racemase is translated from a single transcript and that the protein contains two targeting signals, a mitochondrial sequence at its N terminus and a peroxisomal sequence at its C terminus, explaining the dual subcellular localization of racemase (8, 9). Whether the N-terminal sequence of the racemase is removed after mitochondrial import is not clear. An R-2 mitochondrial matrix processing protease cleavage motif, RX↓XS (28), can be found at position 36–39. Western blot analysis of human liver, however, revealed only one immunosignal corresponding to a protein of 42 kDa (Fig. 3). This would suggest not only a lack of processing but also that the mitochondrial and peroxisomal racemase proteins are translated from the same start methionine.

Apparently different strategies have emerged during evolution in order to economize the targeting of proteins to mitochondria and peroxisomes. One way is through internal translation initiation from a single transcript with the loss of the N-terminal targeting signal, as shown for carnitine acetyltransferases in *Candida tropicalis* (29, 30) and alanine-glyoxylate aminotransferase in the cat and marmoset (31). Another mechanism is based on alternative transcription sites giving rise to mRNAs with a different 5′ end, whereby one encodes a protein containing a mitochondrial presequence as documented for serine-pyruvate/alanine-glyoxylate aminotransferase in the rat (31, 32). The targeting of $\Delta^{3,5}$–$\Delta^{2,4}$-dienoyl-CoA isomerase

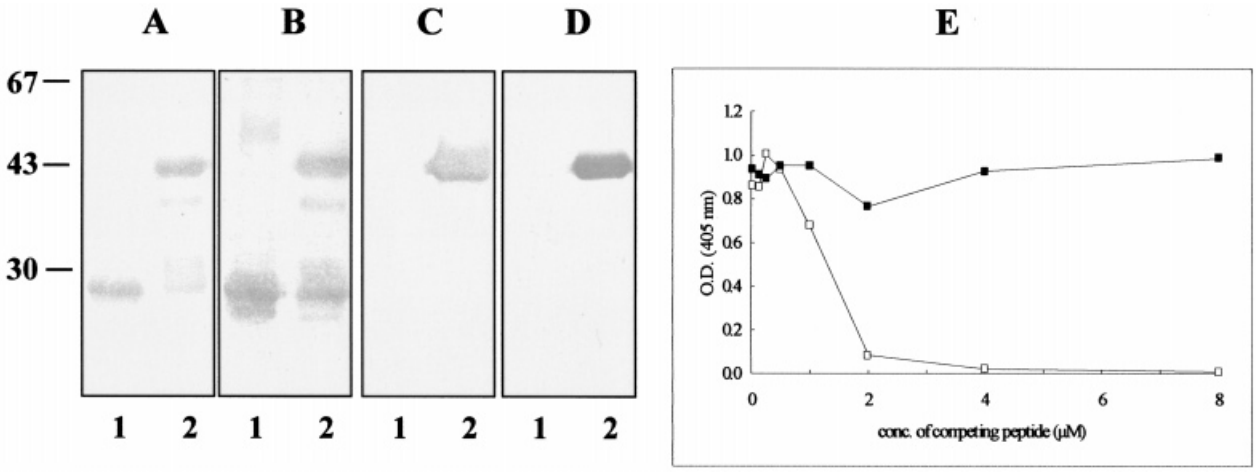

Fig. 4. In vitro interactions of recombinant human racemase with Pex5p. (A–D) Bacterially expressed and purified GST (lane 1) and GST-(Δ1–288)HsRacemase fusion protein (lane 2) were subjected to SDS-PAGE (each 0.5 μg/lane) and blotted onto nitrocellulose membranes. After blocking, the blots were transiently stained with Ponceau (A) and further incubated with rabbit anti-GST (B), biotinylated Pex5p (C), or chicken anti-racemase (D), followed by anti-rabbit or anti-chicken IgG coupled to alkaline phosphatase (B and D) or streptavidin-alkaline phosphatase (C). Phosphatase was revealed by incubation with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium. (E) Wells of microtiter plates were coated with 1 μg of purified GST-(Δ1–288)HsRacemase fusion protein, blocked, and incubated with biotinylated Pex5p in the presence of increasing amounts of competing peptides that end in SKL (open squares) or that do not contain SKL (solid squares). After washing the wells, the amount of retained Pex5p was measured by streptavidin-alkaline phosphatase and *p*-nitrophenyl-phosphate.

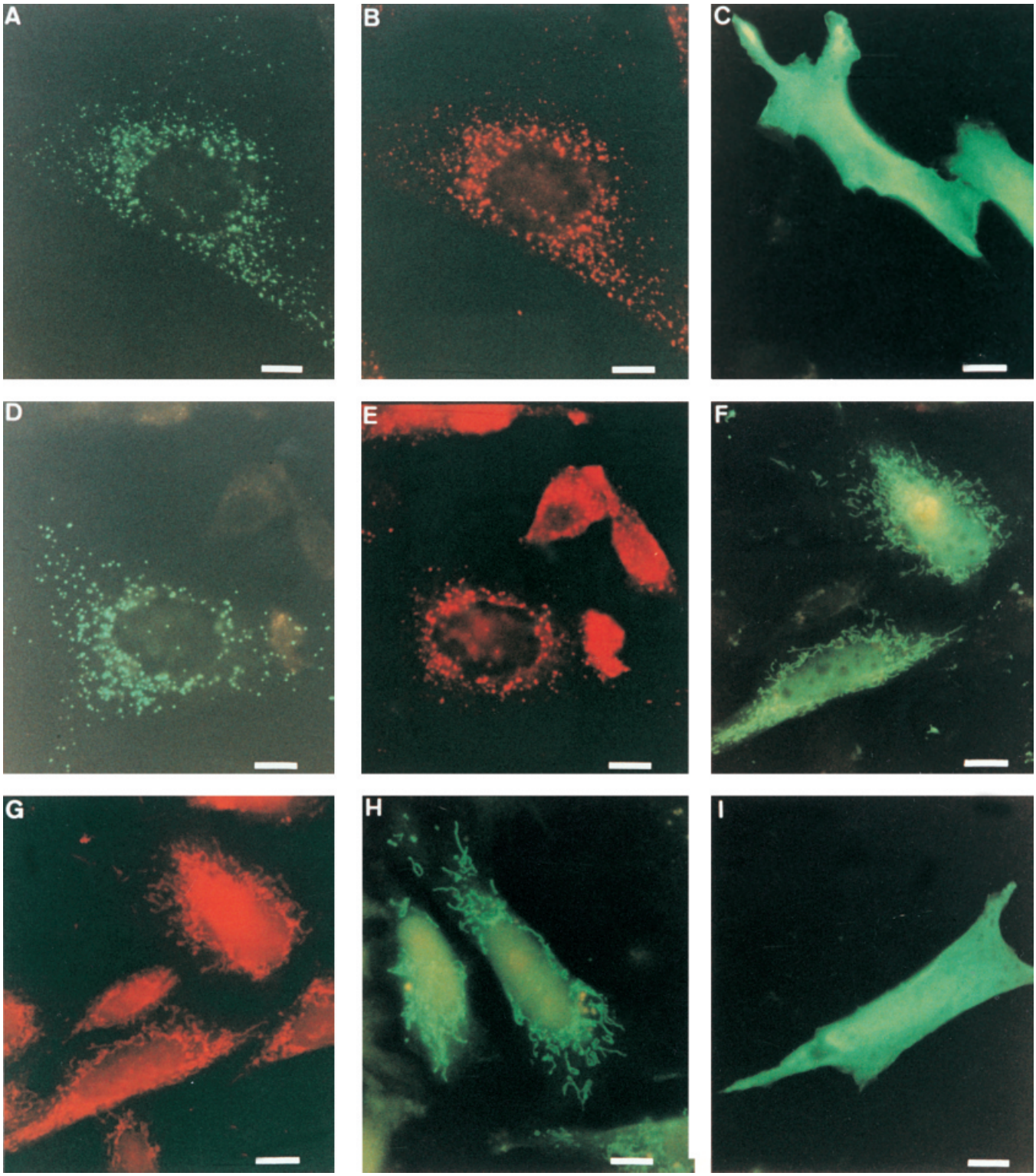

Fig. 5. Targeting of human racemase to peroxisomes and mitochondria. CHO cells were transfected with constructs encoding GFP ending in KVKASL, the C-terminal hexapeptide of human racemase (A and B) or in KVGASL (C) or for GFP fused to the N terminus of full-length racemase (D and E) and analyzed after 48 h by direct fluorescence microscopy (A, C, and D) as described in ref. 22. In (B) and (E), the cells were immunostained with anti-Pex14p. (F, H, and I) Pictures of cells transfected with constructs encoding GFP fused to the C terminus of full-length racemase (F), (Δ1–21) racemase (H), or (Δ1–84) racemase (I) and analyzed by direct fluorescence microscopy. In (G), the cells shown in (F) were stained with Mito-tracker. Scale bar: 10 μm.

(33) and malonyl-CoA decarboxylase (34) in rat and of 3-hydroxy-3-methylglutaryl-CoA lyase in humans (35) resembles that of racemase, except for the mitochondrial processing of the isomerase and the lyase.

The peroxisomal targeting signal of racemase, (K)ASL, is a new PTS1 variant not previously reported but active only when preceded by a lysine residue (or likely a positively charged amino acid). Database searches did not reveal any other putative peroxisomal proteins ending in KASL, either in mammals or in other eukaryotes. Although KASL is thus rather exceptional, the consensus for PTS1 can be broadened to **S**/A/C/K/N-**K**/R/H/Q/N/S-**L**/I in mammals.

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