Cloning and Subcellular Localization of Hamster and Rat Isopentenyl Diphosphate Dimethylallyl Diphosphate Isomerase

A PTS1 MOTIF TARGETS THE ENZYME TO PEROXISOMES*

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To date, isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IPP isomerase; EC 5.3.3.2) is presumed to have a cytosolic localization. However, we have recently shown that in permeabilized cells lacking cytosolic components, mevalonate can be converted to cholesterol, implying that all of the enzymes required for the conversion of mevalonate to farnesyl diphosphate are found in the peroxisome. To provide unequivocal evidence for the subcellular localization of IPP isomerase, in this study, we have cloned the rat and hamster homologues of IPP isomerase and identified the signal that targets this enzyme to peroxisomes. In addition, we also demonstrate that IPP isomerase is regulated at the mRNA level.

The isoprenoid biosynthetic pathway is ubiquitous to all living organisms. A few of the important end products of this complex pathway include: dolichols; vitamins A, D, E, and K; steroid hormones; carotenoids; bile acids; and cholesterol (1). The enzyme isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IPP isomerase); EC 5.3.3.2 plays a crucial role in this pathway by catalyzing the interconversion of isopentenyl diphosphate (IPP) to its highly electrophilic isomer, dimethylallyl diphosphate (2). These two isomers are the building blocks for the successive head-to-tail condensation reactions that result in the synthesis of farnesyl diphosphate (FPP), and ultimately, cholesterol (3).

Recently, it has been shown by our group and others that peroxisomes contain a number of enzymes involved in cholesterol biosynthesis that were previously thought to be cytosolic. Specifically, peroxisomes have been shown to contain acetoacetyl-CoA thiolase (4, 5), 3-hydroxy-3-methylglutaryl coenzyme A reductase (7–9), mevalonate kinase (10, 11), phosphomevalonate kinase (12), mevalonate diphosphate decarboxylase (12), and FPP synthase (13). Both mevalonate kinase and FPP synthase seem to be localized predominantly, if not exclusively, to peroxisomes (11, 13). To date, IPP isomerase is presumed to have a cytosolic localization (1); however, the following three observations have led us to believe that the enzyme is localized to peroxisomes: (i) in permeabilized cells lacking cytosolic components, mevalonate can be converted to cholesterol in equal amounts to that observed in nonpermeabilized cells, therefore suggesting that the cytosol does not contain enzymes necessary for the conversion of mevalonate to FPP (12); (ii) IPP isomerase activity in tissues from patients with peroxisome-deficient diseases (Zellweger and neonatal adrenoleukodystrophy) is 50% of that found in tissues from control patients (13); and (iii) the deduced amino acid sequence from the human isomerase cDNA, which has been recently cloned (14) and characterized (15), contains two putative peroxisomal targeting sequences.

At the C-terminal end of human isomerase is a putative peroxisomal targeting sequence 1 (PTS1) consisting of YRM (single-letter amino acid notation), and at the N-terminal end is a putative PTS2 sequence consisting of HIX(5QL (where X designates any amino acid). The consensus sequence for the PTS1 motif is (S/A/C)(K/H/R)(L/M) (16); however, many tripeptide combinations that do not adhere to this consensus sequence were able to target Saccharomyces cerevisiae malate dehydrogenase to yeast peroxisomes (17). The PTS2 consensus sequence is usually near the N-terminal end of the protein and is the nonpeptide (R/K)(L/V/I)(H/Q)L (18).

In this study, we have cloned the rat and hamster homologues of IPP isomerase and identified the signal that targets this enzyme to peroxisomes. In addition, we demonstrate that IPP isomerase is regulated at the mRNA level in liver from rats treated with compounds known to modulate the levels of cholesterol biosynthesis.

MATERIALS AND METHODS

Materials and General Procedures—Restriction enzymes were purchased from New England Biolabs. Chemically competent Escherichia coli InvAF, electrocompetent E. coli TOP10F, molecular weight markers, TA cloning kit, cDNA synthesis copy kit, and all cloning and expression vectors were obtained from Invitrogen (San Diego, CA).

Microbiological reagents were from Difco. Cholestyramine (Questran) was purchased from Bristol Laboratories. Automated DNA sequencing was performed, and DNA synthetic oligonucleotides were synthesized by the SDSU Microchemical Core Facility (San Diego, CA). The 5' RACE kit was purchased from Life Technologies, Inc. and used according to the provided instructions.

DNA probes were labeled with [32P]dCTP using the Nick Translation kit (Boehringer Mannheim). Zeta Probe GT membrane (used for Northern and Southern analysis), C/P Lift membrane (used for plasmid library screening), and Trans-Blot transfer medium (used for Western analysis) were purchased from Bio-Rad. Western transfer was performed as described (11). Northern and Southern transfers were performed using the Stratagene Posiblot pressure blower and pressure control station (Stratagene) as described by the manufacturer's protocol. Hybridization conditions for Northern analysis, Southern analysis, or plasmid library screening were as follows. Membranes were incubated overnight at a temperature of 50 °C with radiolabeled probe in a solution of 50% formamide, 6% SDS, 100 mM Na2PO4, and 200 mM NaCl; membranes were washed for 15 min, three times, at 55 °C using

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1 The abbreviations used are: IPP isomerase, isopentenyl diphosphate:dimethylallyl diphosphate isomerase; IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; PTS, peroxisomal targeting sequence; PCR, polymerase chain reaction; bp, base pair; UTR, untranslated region; RACE, rapid amplification of cDNA ends.
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0.1% SDS and 0.5 x SSC, and exposed on a PhosphorImager screen (Molecular Dynamics).

Standard molecular biology techniques (19) were routinely used for cloning and restriction endonuclease digestions. Plasmid mini- and large scale preps were performed using QIAprep Spin Miniprep kit and QIAGEN kit, respectively (QIAGEN). DNA or RNA purifications from agarose gels were performed using the Gene Clean kit (Bio 101). Secondary antibodies used in immunofluorescence were from Molecular Probes. Anti-HA monoclonal antibody (12CA5) was from Boehringer Mannheim. All other biochemicals were purchased from Sigma.

DNA Amplification—Where stated, DNA amplification was carried out in a 75 µl reaction mixture containing amplified cDNA from a rat liver cDNA library. Four clones were isolated and used in conjunction with primer E207 (5'-GATACTACGCTCTCTCAGGAAACTATGACC-3') to PCR-amplify a 366-bp fragment containing the PTS1 (5'-GGAGCATGAATTGATTTAGATATGCTGGT-3') as template for the reaction. The primer set for this reaction was 5'-TACATGACCATTGTGCATATGCATGTCGCTGAG (underlined) was used in conjunction with primer T7 (5'-GACGCTCTCTCAGGAAACTATGACC-3') to PCR-amplify a 1428-bp region using the full-length clone, pHamIPPI, as template for the reaction. The product was cloned (pCR2.1), and the resulting vector was termed pCOFRT7. The full-length hamster CHO K1 clone (termed pHamIPPI) was obtained from Dr. Peter Pentchev (National Institutes of Health, Maryland) by cloning the 5' RACE products amplified from CHO K1 poly(A)+ RNA from a combination of 5% cholesterol diet (each in triplicate) were homogenized in homogenization buffer (0.25 M sucrose, 5 mM Tris-HCl, 1 mM EDTA, 0.1% dithiothreitol, 20 mM Tris-HCl, 1 mM EDTA, 0.1% dithiothreitol, 20 mM Tris-HCl, 1 mM EDTA). The resulting cDNA solutions were used to construct an oligo(dT)-primed, reverse transcribed, then subsequently amplified by PCR using the QIAamp tissue kit (QIAGEN), and 15 µg of DNA were digested with HindIII sites of pcDNA3.1. This vector was termed pCOFRT7. The second primer, termed COFRev (5'-GACGCTCTCTCAGGAAACTATGACC-3'), was used in conjunction with primer T7 (5'-GACGCTCTCTCAGGAAACTATGACC-3') to PCR-amplify an 187-bp region using the full-length clone, pHamIPPI, as template for the reaction. The product was cloned (pCR2.1), and the resulting vector was termed pCOFRT7. The first primer, termed COFFor (5'-TACATGACCATTGTGCATATGCATGTCGCTGAG (underlined) was used in conjunction with primer T7 (5'-GACGCTCTCTCAGGAAACTATGACC-3') to PCR-amplify a 1428-bp region using the full-length clone, pHamIPPI, as template for the reaction. The product was cloned (pCR2.1), and the resulting vector was termed pCOFRT7.

Liver Homogenate Preparation and Western Blot Analysis—Livers from five 5% cholesterol plus mevinolin and, as a control, livers from rats fed a cholestyramine plus mevinolin diet (each in triplicate) were homogenized in homogenization buffer (0.25 M sucrose, 5 mM Tris-HCl, 1 mM EDTA, 0.1% dithiothreitol, 20 mM Tris-HCl, 1 mM EDTA), 0.1% dithiothreitol, 20 mM Tris-HCl, 1 mM EDTA) and 10% aprotinin, 10 µg/ml cycloheximide, 0.1 µl dithiothreitol, 20 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml chymostatin, 1 µg/ml pepstatin A, and 0.1 µl phenylmethylsulfonfluoride, pH 7.5, at 4°C (20). The protein concentration of the homogenate was determined by the BCA method (Pierce Chemical Co.) using bovine serum albumin as a standard, and 200 µg of protein from each sample were electrophoresed on a 12% SDS-polyacrylamide gel as described (20). The gel was transferred to nitrocellulose, and immunoblot analysis was performed as described previously (11). The anti-therioredoxin-isomerase antisera was added at a 1:1000 dilution, and the secondary antibody (horseradish peroxidase protein A conjugate) was used at a 1:5000 dilution. The blot was then processed using the ECL kit (Amersham).

Internal Epitope Tagged Expression Vector Construction—Two DNA oligonucleotides were used to introduce the HA epitope into the hamster isomerase. The first primer, termed COFRev (5'-GACGCTCTCTCAGGAAACTATGACC-3'), was used in conjunction with primer T7 (5'-GACGCTCTCTCAGGAAACTATGACC-3') to PCR-amplify a 1428-bp region using the full-length clone, pHamIPPI, as template for the reaction. The product was cloned (pCR2.1), and the resulting vector was termed pCOFRT7. The second primer, termed COFFor (5'-TACATGACCATTGTGCATATGCATGTCGCTGAG (underlined) was used in conjunction with primer T7 (5'-GACGCTCTCTCAGGAAACTATGACC-3') to PCR-amplify a 1428-bp region using the full-length clone, pHamIPPI, as template for the reaction. The product was cloned (pCR2.1), and the resulting vector was termed pCOFRT7. The first primer, termed COFRev (5'-GACGCTCTCTCAGGAAACTATGACC-3'), was used in conjunction with primer T7 (5'-GACGCTCTCTCAGGAAACTATGACC-3') to PCR-amplify a 1428-bp region using the full-length clone, pHamIPPI, as template for the reaction. The product was cloned (pCR2.1), and the resulting vector was termed pCOFRT7. The second primer, termed COFFor (5'-TACATGACCATTGTGCATATGCATGTCGCTGAG (underlined) was used in conjunction with primer T7 (5'-GACGCTCTCTCAGGAAACTATGACC-3') to PCR-amplify a 1428-bp region using the full-length clone, pHamIPPI, as template for the reaction. The product was cloned (pCR2.1), and the resulting vector was termed pCOFRT7.
RESULTS AND DISCUSSION

Cloning of Rat and Hamster IPP Isomerase—Based on the cDNA sequence of human IPP isomerase (14), two oligonucleotide primers were constructed and used in RT-PCR on rat liver poly(A)^+ RNA to amplify 667 bp of the isomerase coding region. This PCR product was used as a probe to screen an oligoA^+ cDNA library made from rat liver RNA isolated from a rat fed a C^+M diet. These two drugs up-regulate mRNA levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase and a number of other enzymes involved in the cholesterol biosynthetic pathway (22). A total of approximately 500,000 colonies were screened, and four individual clones were isolated and sequenced. All four clones had 5′ UTRs of different lengths, varying from 72 to 385 nucleotides (data not shown). To identify the size of the longest 5′ UTR on poly(A)^+ RNA isolated from the liver of a C^+M-fed rat. The PCR product indicated that the longest 5′ UTR was 385 nucleotides, giving a size of 1182 bp for the complete cDNA of rat IPP isomerase (data not shown).

The full-length cDNA for hamster IPP isomerase was isolated utilizing 5′ and 3′ RACE. The experiments were performed using poly(A)^+ RNA isolated from CHO K1 cells. Eight individual, overlapping, partial clones were isolated and sequenced (data not shown).

The deduced amino acid sequences from both the rat and hamster cDNAs were compared with the deduced amino acid sequence of human IPP isomerase (Fig. 1). The following results were obtained: (i) hamster isomerase has an 89.4% identity and a 95.6% similarity to human isomerase; (ii) rat isomerase has an 86.3% identity and a 92.5% similarity to human isomerase; (iii) hamster and rat isomerases have a 91.6% identity and a 95.6% similarity to human isomerase; and (iv) hamster and rat isomerases share 91.6% identity (96.0% similarity), indicating a high evolutionary conservation of the protein. Putative PTS motifs are denoted by a line. * catalytically important residues. GenBank accession numbers for the rat and hamster cDNA sequences are AF003835 and AF003836, respectively.

Immunofluorescence Microscopy—Cells on coverslips were washed in PBS and fixed in 3.0% paraformaldehyde in PBS for 15 min. Cells were permeabilized with 1% Triton X-100 in PBS for 5 min and then washed with 0.1% Tween 20 in PBS (also used for subsequent washes). A mixture of rabbit anti-catalase and mouse anti-HA (both at a final dilution of 1:50) was applied to the coverslips and incubated for 60 min. The cells were washed extensively, and the coverslips for 60 min. The cells were washed extensively, and the coverslips were mounted on microscope slides for observation with a Nikon fluorescence microscope.

Fig. 1. Deduced amino acid comparison of hamster, rat, and human IPP isomerases. Black boxes represent nonconserved amino acids, and shaded boxes represent conserved amino acids. All other amino acids are identical. Hamster and human sequences share 89.4% identity (95.6% similarity), rat and human sequences share 86.3% identity (92.5% similarity), and rat and hamster sequences share 91.6% identity (96.0% similarity), indicating a high evolutionary conservation of the protein. Putative PTS motifs are denoted by a line. * catalytically important residues. GenBank accession numbers for the rat and hamster cDNA sequences are AF003835 and AF003836, respectively.
Regulation of IPP isomerase protein levels by diet. Homogenates were prepared from the livers of nine rats; CH, diet supplemented with 2% cholesterol; C, control diet; and C+M, a diet supplemented with 5% cholesteryamine + 0.1% mevinolin. Two hundred μg of protein from each homogenate was electrophoresed on a 12% SDS-polyacrylamide gel. The proteins in the gel were transferred to nitrocellulose, and the membrane was blotted using anti-thioredoxin-IPP isomerase antiserum. Arrow, approximate molecular weight of the band up-regulated by the C+M diet.

Southern blot hybridization of 32P-labeled IPP isomerase. Rat genomic DNA was digested with BamHI, NotI, and XbaI, and the digests (15 μg of DNA/lane) were subjected to electrophoresis in a 0.8% agarose gel. The DNA was transferred to a nylon membrane and hybridized with a radiolabeled 180-bp rat isomerase fragment of the coding region of rat IPP isomerase. Dashes, positions of the size markers.

Eukaryotic expression vector constructs of IPP isomerase. A, diagrammatic illustration of internal HA epitope tag of IPP isomerase. Amino acids of the native isomerase (pHAMlIPP) from residues 136 to 143 were replaced with amino acids encoding the HA epitope, resulting in the eukaryotic expression vector construct, pHS0HA. B, the putative PTS1 of pHS0HA was deleted by the addition of a stop codon, resulting in expression vector pHS0HA-PTS1.

IPP Isomerase Is Present as a Single Copy Gene in the Rat Genome—To identify whether IPP isomerase is present as a single copy gene, a Southern blot was performed using rat genomic DNA. The blot was probed with a 180-bp rat isomerase fragment, chosen due to its high degree of conservation among several species (15). The Southern blot revealed a single band hybridizing in each lane (Fig. 4), providing evidence that rat IPP isomerase is present as a single copy gene. These data further suggest that the two transcripts (2.4 and 1.1 kb) visualized by Northern analysis (Fig. 2A) originated from a single gene.

IPP Isomerase Is Targeted to Peroxisomes by a PTS1 Motif—Because all of the mammalian isomerases have putative PTS1 and PTS2 motifs, we first constructed a eukaryotic expression vector containing the full coding sequence of hamster IPP isomerase with an internal HA epitope tag. This vector was termed pHS0HA (Fig. 5A). An internal HA tag was chosen instead of C- or N-terminal HA tags so as not to disrupt the potential PTS1 or PTS2 function.

To determine in which subcellular compartment IPP isomerase is localized, we transfected pHS0HA into control human fibroblast cells. The cells were then simultaneously immunolabeled with anti-catalase antibody (Fig. 6A) and anti-HA antibody (Fig. 6B). The immunofluorescence pattern for catalase was superimposable over the pattern obtained with the HA antibody. Similar results were obtained in CHO cells when labeled with anti-catalase antibody (Fig. 6C) and anti-HA antibody (Fig. 6D). These results show that hamster IPP isomerase is colocalized with catalase to peroxisomes.

To determine which of the PTS targeting signals are used by the hamster isomerase, we used two distinct cell lines derived from patients with peroxisomal disorders. One cell line, BRO-T, shown to be deficient in the peroxisomal import of only PTS2 proteins, was from a patient diagnosed with rhizomelic chondrodysplasia punctata, belonging to complementation group 11 (23). The other cell line, FAIR-T, shown to be deficient in the peroxisomal import of both PTS1 and PTS2 proteins, was from a patient diagnosed with Zellweger syndrome, belonging to complementation group 2 (23). To first determine if the putative PTS2 is responsible for peroxisomal import, the construct...
peptide was deleted (Fig. 5B). This deletion construct was transfected into the PTS2-deficient cell line BRO-T. The immunofluorescence pattern was consistent with peroxisomal labeling when anti-catalase antibody was used (Fig. 7A), whereas a cytosolic labeling pattern was obtained when the anti-HA antibody was used (Fig. 7B). Thus, these data show that the HRM tripeptide is necessary for the targeting of IPP isomerase to peroxisomes.

The HRM tripeptide is present at the C terminus of both the rat and hamster homologues of IPP isomerase. However, the human homologue at its C terminus has a YRM tripeptide (Fig. 1).

The PTS1 consensus sequence of (S/A/C)(K/H/R)(L/M) was derived from extensive mutational analysis, where peroxisomal proteins from other organisms or nonperoxisomal proteins were used as reporters. Because of the formulation of this consensus sequence, more peroxisomal proteins have been identified, the PTS1-like tripeptide of which does not fit this exact sequence. However, it has been demonstrated recently that many amino acid substitutions can be made at the first position of a homologous protein without compromising the PTS1 function (17). Thus, this strongly suggests that human isomerase is also targeted to peroxisomes by a PTS1, because the RM dipeptide meets the consensus sequence criteria.

The peroxisomal enzymes required for conversion of mevalonate to FPP (i.e., mevalonate kinase, phosphomevalonate kinase, mevalonate diphosphate decarboxylase, isopentenyl diphosphate isomerase, and FPP synthase) have now been all cloned and sequenced. Four of the five enzymes, mevalonate kinase (25), phosphomevalonate kinase (26), mevalonate diphosphate decarboxylase (27), and isomerase (14), contain a conserved putative PTS1 or PTS2, supporting the concept of a targeted transport into peroxisomes. This is the first study to unequivocally demonstrate that IPP isomerase, one of the five enzymes involved in the conversion of mevalonate to FPP, uses a PTS1 for peroxisomal targeting. FPP synthase does not contain a currently identifiable PTS1 or PTS2, yet it is selectively localized to peroxisomes. It seems likely that targeting to peroxisomes must include alternative mechanisms, not yet defined.

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