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UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

**CHARACTERIZATION OF IDI2, A SECOND ISOPENTENYL
DIPHOSPHATE IN MAMMALS**

A dissertation in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Biology

by

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2006

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2006

Dedication

To my family
To Shauna, who is my tenderness
To Aimee, who is my strength
To Ashly, who is my wisdom
To Sam, who is my loving kindness
To Scott, who is my joy
To Mom, who is my inspiration and my courage
You have stood by me and weathered my storms
You have been the colors in my rainbows
I would not be who I am if it weren't for your unending love, laughter and
support.
With profound gratitude
These pages and all they imply
Are dedicated to you.

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Characterization of IDI2, a Second Isopentenyl Diphosphate Isomerase in Mammals. Manuscript submitted. JBC. Clizbe D, Owens M, Shackelford J, Krisans SK.

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Isopentenyl Diphosphate Isomerases in Human and Mouse: Evolutionary Analysis of a Mammalian Gene Duplication. J Mol Evol. 2003 Sep;57(3):282-91. Breitling R, Laubner D, Clizbe D, Adamski J, Krisans SK.

ABSTRACT OF THE DISSERTATION

CHARACTERIZATION OF IDI2, A SECOND ISOPENTENYL DIPHOSPHATE
IN MAMMALS

BY

DAUN BARR CLIZBE

DOCTOR OF PHILOSOPHY IN BIOLOGY

UNIVERSITY OF CALIFORNIA, SAN DIEGO, 2006
SAN DIEGO STATE UNIVERSITY, 2006

PROFESSOR SKAIDRITE KRISANS, CHAIR
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Isoprenoids and isoprenoid derived compounds play an essential role in all living systems. All isoprenoids are derived from IPP and its isomer DMAPP. The isomerization reaction is catalyzed by isopentenyl diphosphate isomerase (IDI). Here we report the isolation of a full length cDNA coding for a novel IDI (IDI2) in humans. Chromosomal localization studies as well as significant sequence similarity at the nucleotide and amino acid level indicate that the novel isomerase is the product of a duplicated gene. In plants, this central reaction of isoprenoid biosynthesis is catalyzed by various highly conserved isozymes that differ in expression pattern and subcellular localization. In humans, real time PCR data has shown that IDI2 is expressed

at high levels only in skeletal muscle. These results have been confirmed by Northern analysis. We propose that after the initial duplication IDI2 underwent a short phase of apparently random change, during which its active center became modified. Molecular modeling shows that despite a cysteine to serine change within the critical active site, IDI2 is likely to catalyze the isomerization of IPP to DMAPP. We have generated expression constructs in *S.cerevisiae* for IDI2. Our results indicate that, under in-vitro, assay conditions, IDI2 has the ability to catalyze the isomerization of ^{14}C -IPP to ^{14}C -DMAPP. Importantly, IDI2 can complement isomerase function in an *idi1* deficient yeast strain. Kinetics on partially purified IDI2 indicate the novel isozyme has a maximal relative specific activity of $1.2 \times 10^{-1} \pm 0.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$ at pH 8.0 with a K_m^{IPP} value of 22.8 μM IPP, values significantly lower than those reported for human IDI1. In addition, isomerase activity in purified IDI2 is affected differently by flavin dinucleotides and has a different requirement for the divalent metal Zn^{2+} than IDI1. We show that both IDI1 and IDI2 are localized to the peroxisome by a PTS1 dependent pathway. Moreover, while our results indicate that IDI1 is regulated by the SREBP pathway we show that IDI2 is regulated independently by a mechanism that may involve $\text{PPAR}\alpha$.

Introduction

Isoprenoid biosynthetic pathway: Overview

Isoprenoids and their derivatives perform vital roles in all living systems. Over 35,000 naturally occurring isoprenoid compounds have been identified with new compounds continually being discovered. Hopinoids an ancient form of isoprenoid have been found in sediments dating as far back as 2.5 billion years (1). They provide a necessary function in the organization of many biological systems including membrane structure, signal transduction, and redox chemistry. Several of the important end products of the isoprenoid biosynthetic pathway include : prenylated proteins, dolichols, vitamins A, D, E and K, steroid hormones, carotenoids, bile acids and cholesterol (2).

Isoprenoids can be divided into two major groups. The most abundant form, the terpenoids, are the products derived solely from C_5 -units. The less common meroterpenoids are formed by the reaction of one intermediate from another biosynthetic pathway. All isoprenoids are derived from the 5 carbon isoprene defined by isopentenyl diphosphate (IPP) and its highly electrophilic isomer dimethylallyl diphosphate (DMAPP). In eukarya and archaea, where the pathway is well characterized, IPP is synthesized from three molecules of acetyl-CoA by the classical mevalonate pathway (figure 1).

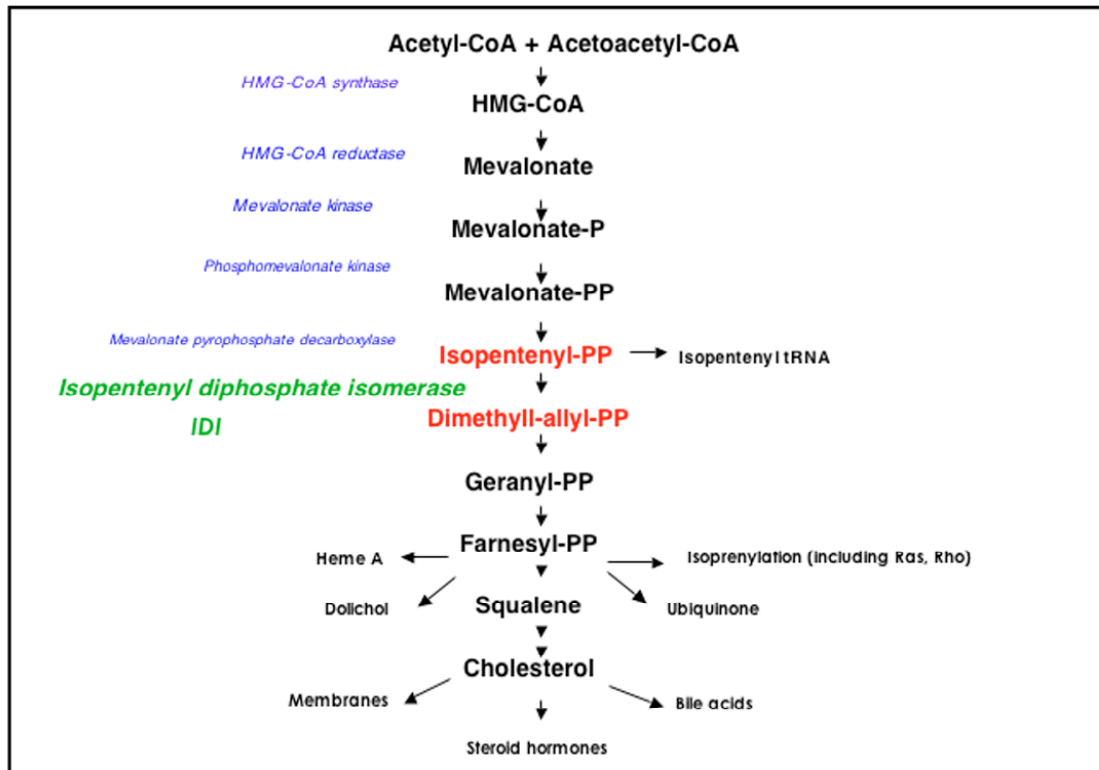


Figure 1. Overview of the isoprenoid biosynthetic pathway. Synthesis of isoprenoids and ultimately cholesterol are formed through multiple steps beginning with the condensation of acetyl-CoA and acetoacetyl CoA. The first committed step of the pathway is the conversion of HMG-CoA to mevalonate by HMG-CoA Reductase (HMGCR). Mevalonate is converted into the isoprene IPP which is isomerized into the more electrophilic isomer DMAPP via the enzyme isopentenyl diphosphate isomerase (IDI). These 5 carbon isoprenes condense to form the C₁₀ geranyl pyrophosphate (GPP). A further addition of IPP results in C₁₅ farnesyl pyrophosphate (FPP). FPP is the branch point between sterol and non-sterol synthesis. The head to head condensation of two FPP molecules result in the formation of the sterol precursor, squalene. cyclization of squalene leads ultimately to the formation of cholesterol.

Isoprenoid pathway products

β -Hydroxymethylglutaryl coenzyme A (HMG-CoA) ultimately derived from the condensation of acetyl-CoA is converted to mevalonate by the enzyme HMG-CoA reductase (HMGCR) in a NADPH dependent reaction. Mevalonate is subsequently phosphorylated by the enzyme mevalonate kinase (MK) to yield 5-phosphomevalonate.

5-phosphomevalonate is then phosphorylated and decarboxylated to form the 5 carbon isopentenyl pyrophosphate (IPP). IPP can be converted to its highly electrophilic isomer dimethallyl diphosphate (DMAPP) by the enzyme isopentenyl diphosphate isomerase (IDI). This critical activation step provides substrate for subsequent head to tail condensation reactions to form C₁₀ geranyl pyrophosphate (GPP). GPP is the precursor for synthesis of all monoterpenes. Condensation of another molecule of IPP by the enzyme FPP synthase yields the C₁₅ farnesyl pyrophosphate (FPP). Synthesis of FPP represents a branch point for synthesis of sterols and longer-chain non-sterol species. The head to head condensation of two molecules of FPP by the enzyme squalene synthase yields the sterol precursor squalene. Subsequent cyclization steps lead to sterol synthesis. Geranylgeranyl pyrophosphate (GGPP) synthase catalyzes the addition of IPP to FPP to form C₂₀ GGPP. GGPP is the precursor in plants for carotenoids, diterpenes and chlorophylls and in some cases used for synthesis of longer-chain products (3).

Isoprenoids: a regulatory role

A class of GPP derivatives formed primarily in plants, the monoterpenes have recently received a great deal of attention for their potential as chemopreventive as well as chemotherapeutic agents. Recently the monoterpenes limonene and perillyl alcohol have been shown to inhibit or induce complete regression of growth of mammary and pancreatic tumors through inhibition of ras oncogene (3). Many derivatives of FPP have also been shown to have therapeutic effects. Plant-derived sesquiterpenes have

shown numerous biological activities including inhibitors of nuclear factor kappaB (4) anti-tumor agents (5) and anti-HIV agents (6) . Several lines of evidence suggest that FPP and its cognate alcohol, farnesol (FOH) may be non-sterol regulators of HMGCR. Recent studies have shown that there is an interconversion of between FPP and farnesol (FOH) in cells (7). To date it remains controversial whether FPP or FOH is responsible for HMGCR regulation. A role for FOH has been suggested in studies in CHO cells using an FPP analog that induced HMGCR degradation, this effect was reversed by addition of endogenous FOH (7). However, studies using a mutant yeast strain lacking the farnesyl pyrophosphatases LPP1 and DPP1 still retained functional regulation of HMGCR degradation indicating a role for FPP (8)

Isoprenoid regulation of prenylated proteins

FPP and GGPP also function as isoprene donors in the isoprenylation of proteins. Farnesylated proteins include Ras superfamily G-proteins as well as tyrosine phosphatases. Farnesylation is catalyzed by protein farnesyltransferase which recognizes the CysAAX motif at the C-termini of substrate proteins and transfers a farnesyl group forming a thioether bond. This heterodimeric enzyme is conserved from yeast to human, and their genes have been identified in a variety of organisms (9). Recent studies demonstrate the strong link between isoprenoid species FPP and GGPP and regulation of Ras and Ras-related proteins (10). Initial studies indicated that mevalonate depletion results in upregulation of Ras and Ras-related proteins including Rap1A, RhoA, and RhoB on multiple levels including transcriptional,

translational and posttranslational levels (10). Later works indicated that some structurally related retinoids including all-trans-retinoic acid and 9-cis-retinoic acid also display slight agonist properties. However, recently, studies have provided evidence for direct roles of FPP and GGPP in regulating Ras and Ras-related protein transcriptional and post-transcriptional events (10).

Transcription factor interaction

Farnesol was shown to activate the farnesoid X receptor (FXR), an orphan member of the nuclear hormone receptor superfamily (11). In early studies FXR, cloned from rat, was shown to have high identity with the insect ecdysone receptor (EcR) (11). EcR is known to form a complex with the insect homolog of RXR, and to bind to DNA containing an ecdysone response element (11). Earlier studies had indicated that FXR:RXR heterodimers bind to an ecdysone response element (11). The addition of either farnesol or juvenile hormone III, a structurally related isoprenoid, to mammalian cells activated a ecdysone response element reporter gene in an FXR and RXR dependent fashion (11). Furthermore, analogs of JH 111 including methoprene, methoprene acid and hydroprene acid have been shown to activate and in some cases directly bind to the nuclear transcription factor, liver X receptor (LXR) (12). In contrast, studies have demonstrated that GGOH acts as an inhibitor of LXR. Additional studies have shown that GGPP also acts as an LXR antagonist by a mechanism of interference with LXR co-activators (13). It has been shown that through LXR inhibition, GGPP can downregulate expression of the ATP-binding cassette transporter A1 (ABC-A1) (13).

Recent studies have revealed a novel role for FOH in peroxisome proliferator activated receptor α (PPAR α) regulated transcription of differentiation-specific genes in epidermal keratinocytes (14). PPAR- α mRNA levels and PPAR- α activity were increased in these cells following treatment with FOH.

Regulation of the Isoprenoid biosynthetic pathway

The majority of genes involved in the isoprenoid pathway are regulated at the transcriptional level by the sterol regulatory element-binding protein (SREBP) family of transcription factors (15). SREBPs are part of a larger family of basic-helix-loop-helix-leucine-zipper (bHLHLZ) transcription factors. SREBPs are synthesized as membrane bound precursors that require a two-step proteolytic processing in order to be released from their amino terminal domains. Proteolytic cleavage allows them to translocate to the nucleus where they bind to a specific DNA sequence : the sterol regulatory element (SRE), thereby activating target genes in a sterol-regulated manner.

Three forms of SREBP have been characterized SREBP 1a,-1c and -2. Through studies in transgenic and knock-out mouse models it has been shown that SREBP2 specifically activates genes involved in cholesterol biosynthesis . SREBP1a plays a role in activating transcription of genes involved in fatty acid synthesis and cholesterol biosynthesis (16, 17)

SREBP transcription factors are regulated at three major levels: transcription, proteolytic cleavage of SREBP precursors and post-translational modification of the nuclear form of SREBP (nSREBP). SREBP-1a and

SREBP-2 are primarily regulated at the level of precursor cleavage while studies indicate that SREBP-1c is primarily regulated at the transcriptional level.

SREBP activation by proteolytic cleavage proceeds through successive steps (figure 2). Following SREBP mRNA translation, precursor proteins are held in the ER by association with SREBP cleavage activating protein (SCAP). In the presence of cholesterol within the ER, the SREBP/SCAP complex is bound by insulin induced gene (insig) in the ER. When sterol levels are low the SREBP-SCAP complex moves to the Golgi bound to COPII where cleavage by two functionally distinct proteases occur. Site 1 (S1P) and site 2 protease (S2P) sequentially cleave the precursor protein releasing nSREBPs into the cytoplasm.

SREBPs have a unique specificity for their target genes. Unlike other members of the bHLH-LZ family of transcription factors which contain a conserved arginine in their basic domain SREBPs have a tyrosine at this position. Substitution of this residue allows SREBPs to bind on both E-boxes (5'-CANNTG-3') but also SRE sequences (5'-TCACNCCAC-3') (18).

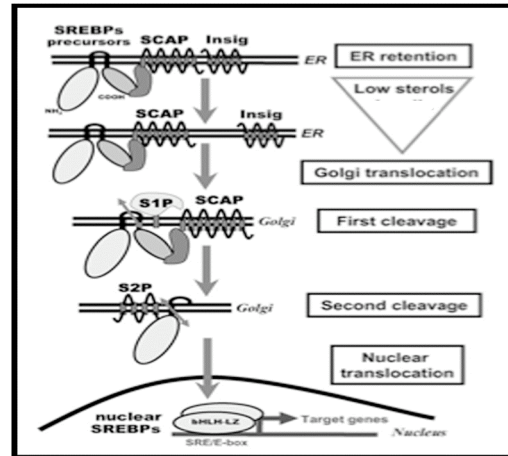


Figure 2. SREBPs activation by proteolytic cleavage. SREBPs are transcription factors synthesized as inactive precursors bound to the membranes of the ER. They are tightly associated with SCAP escorting protein. SCAP interacts with the Insig proteins which results in the complex being retained in the ER. Upon appropriate conditions, (low sterols) the interaction between Insig and SCAP decreases and allows SCAP to escort SREBPs to the Golgi where S1P cleaves SREBP at the luminal site. S2P then cleaves releasing the NH₂-terminal SREBP domain (nSREBP). This domain containing the bHLH-LZ region is then translocated to the nucleus where it will bind its target genes on SRE or Ebox sequences as a homodimer. (adapted from Kim et al 1995)

Isoprenoid biosynthetic enzymes in peroxisome

Peroxisomes are single membrane bound organelles that are ubiquitous throughout eukaryota. Their wide ranging functions are largely dependent upon the species and cell type and are under the influence of environmental and/or developmental conditions (19). Peroxisomes are responsible for decomposition of toxic cellular hydrogen peroxide by the enzyme catalase, an essential reaction carried out by virtually all peroxisomes. In addition, peroxisomes are involved in the β - and α -oxidation of fatty acids, ether lipid synthesis and oxidation of bile acids and cholesterol (20,21).

Enzymes required for the conversion of mevalonate to FPP have been shown to localize exclusively to the peroxisome (20,21)(figure 3). Several of

the enzymes including HMGCR, acetoacetyl-CoA thiolase and HMG-CoA synthase are known to be localized to multiple compartments. The enzymes phosphomevalonate kinase (PMvK), mevalonate diphosphate decarboxylase (MPD) and isopentenyl diphosphate isomerase (IDI) were previously thought to localize to the cytosol. However, in experiments using intact and digitonin treated CV-1 cells enzyme activities were shown to be equal (22). Digitonin selectively permeabilizes plasma membranes leaving organellar membranes intact, thus, indicating the enzymes were contained within membrane bound organelles and were not cytosolic. Additional studies using immunofluorescence of tagged proteins cloned from rat or hamster indicated that these enzymes colocalized with peroxisomal marker enzymes (23, 24). Recently, however, human homologs of MVA kinase, PMvK and MvPP decarboxylase were shown to localize to the cytosol in human fibroblasts (25,26,27). It was proposed that localization of these enzymes was species specific. Recently studies in our lab using immunofluorescence analysis of over-expressed proteins in human cells confirm previous findings that MVA kinase, IDI and FPPS and PMvK are peroxisomal (Kovacs et al manuscript in press).

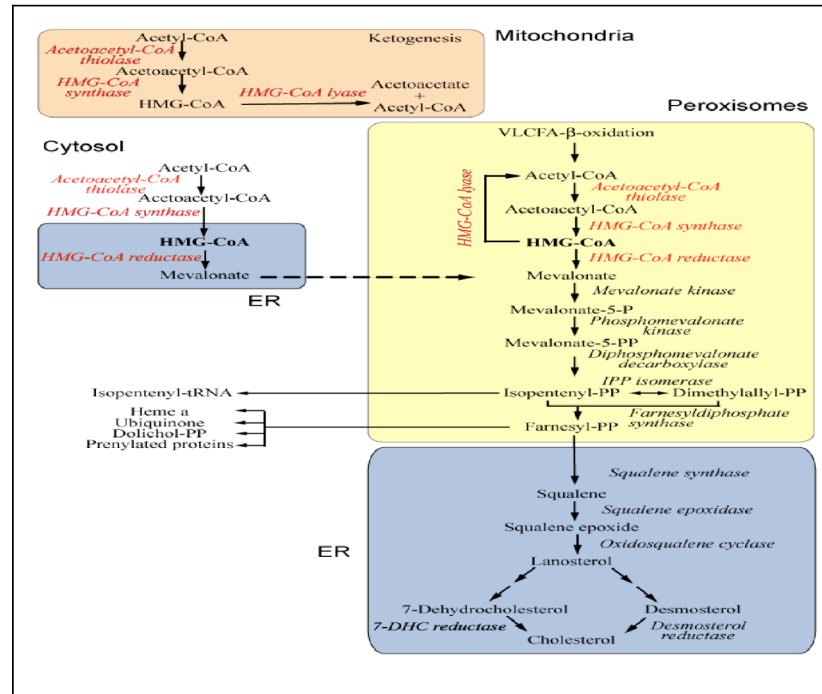


Figure 3. Compartmentalization of enzymes of the isoprenoid/cholesterol biosynthetic pathway. Peroxisomes contain all enzymes for the conversion of acetyl-CoA to FPP. With the exception of HMGCR these enzymes contain peroxisomal targeting signals. The conversion of acetyl-CoA to HMG-CoA also occurs in the cytosol. The further conversion of HMG-CoA to mevalonate occurs both in the ER and peroxisomes. A single gene encodes the mitochondrial and peroxisomal acetoacetyl-CoA thiolase. Similarly both the cytosolic and peroxisomal HMG-CoA synthase are encoded by a single gene. (Kovacs et al. 2002. review.)

Peroxisomal matrix proteins are imported to the peroxisome from the cytosol through the recognition of targeting signals by specific receptor proteins. To date, two different peroxisomal targeting signals have been identified, a C-terminal peroxisomal targeting signal 1 or PTS1 and an N-terminal PTS2 located within the first 30 amino acids of a peroxisomal protein. PTS1 is a carboxyl-terminal tripeptide with the consensus sequence (S/C/A)(K/R/H) (L/M).

However, it has been shown that the efficiency of various tripeptide combinations to function as PTS1s is enhanced by adjacent "accessory" sequences.

A small subset of peroxisomal matrix proteins is targeted by PTS2, located at the N-terminus of proteins at variable distances from the initiating methionine residue and is sometimes cleaved inside the peroxisome by a specific protease (28). The consensus PTS2 first defined as a nonapeptide with the sequence -RLX₅(H/Q)L-, was later modified to - (R/K)(L/V/I)X₅(H/Q)(L/A)- (28). There is some evidence that the PTS2 may actually rely less on a required sequence of amino acids than a yet undefined structural or charge based motif (29). Proteins lacking identifiable PTS1 or PTS2 have been shown to import into the peroxisomal matrix. These proteins are thought to import via either the use of an yet unidentified PTS1 or PTS2 or a unique PTS3 pathway or possibly by "piggybacking" as part of a complex with other PTS-containing proteins (30, 31). The majority of the enzymes involved in synthesis of FPP from mevalonate contain either a PTS1 or PTS2 (Table 1).

Table 1.

Protein name	Sequence of carboxy terminus including PTS1 motif	Organism	Accession number
Acetoacetyl-CoA thiolase	GGGASAMLIQKL	<i>Hs</i>	D90228
	GGGASALLIEKL	<i>Mm</i>	BC024763
	GGGASAVLIEKL	<i>Rn</i>	D13921
Phosphomevalonate kinase	LENLIEFIRSRL	<i>Hs</i>	BC007694
	LEHLLGFIQAKL	<i>Mm</i>	AK003607BC
	NLLEFIHAKLQR	<i>Rn</i>	086349
Isopentenyl-PP isomerase	NQFVDHEKIYRM	<i>Hs</i>	AF271720
	SPFVDHEKIHRL	<i>Mm</i>	BC004801
	SPFVDHEKIHRL	<i>Rn</i>	AF003835
PTS2 sequences			
HMG-CoA synthase	SVKTNLMQL	<i>Hs</i>	BC000297
	SVKSNLMQL	<i>Mm</i>	BC029693
	SVKSNLMQL	<i>Rn</i>	X52625
Mevalonate kinase	KVILHGEHA	<i>Hs</i>	M88468
	KVILHGEHA	<i>Mm</i>	BC005606
	KVILHGEHA	<i>Rn</i>	M29472
Mevalonate-PP decarboxylase	SVTLHQDQL	<i>Hs</i>	U49260
	SVTLHQDQL	<i>Mm</i>	AJ309922
	SVTLHQDQL	<i>Rn</i>	U53706
Farnesyl-PP synthase	NSDVYAQE	<i>Hs</i>	J05262
	KLDAYNQE	<i>Mm</i>	BC048497
	KLDVHNQE	<i>Rn</i>	M34477
Consensus PTS1	(S/C/A)(K/R/H)(L/M)		
Consensus PTS2	(R/K)(L/V/I)X5(H/Q)(L/A)		

Peroxisomal matrix protein import

Peroxisomal proteins are divided into two groups according to their final destination either matrix or membrane within the peroxisome. Peroxisomal membrane and matrix proteins are imported post-translationally via their own import pathway. All peroxisomal proteins are encoded by nuclear genes known as peroxins (PEX). To date over 25 PEX genes have been identified (32).

The import of matrix proteins proceeds in a well characterized multi-step process including the binding of the cargo to the receptor,

the cargo-receptor complex docking at the target membrane, translocation of the cargo or cargo-receptor complex, and finally export of the receptor. The import of matrix proteins is dependent upon two well characterized targeting signals, PTS1 and PTS2, their largely cytosolic receptors pex5p and pex7p, as well as at least ten membrane-bound peroxins involved in the transport of folded matrix proteins across the peroxisomal membrane (33).

Matrix proteins are synthesized on free ribosomes in the cytosol and are bound by the peroxisomal targeting sequence receptors pex5p and pex7p. As mentioned previously Two types of targeting sequences have been identified. The most abundant, peroxisomal targeting signal type 1 (PTS1) consists of a conserved tripeptide at the C-terminus of the protein and a less well-conserved upstream region (34, 35). Peroxisomal targeting signal type 2 (PTS2) located near the N-terminus consists of a degenerate nine residue signal located internally or near the N-terminus and in some cases is cleaved after import. (36,37,38).

The PTS1 receptor pex5p contains seven tetratricopeptide repeat (TPR) domains, shown to be essential for PTS1 binding (39) Six out of the seven TPR domains are shown to interact directly with the C-terminal PTS1 tripeptide, another, TPR4, is involved in maintaining the correct structural alignment (40,41).

In most cases, proteins that possess the peroxisomal targeting signal PTS1 bind the pex5p receptor whereas the proteins that possess PTS2 bind to the cytosolic receptor pex7p. The receptor-cargo complex then docks with the

mammalian proteins pex13p, pex14p and pex17p proteins in *S cerevisiae* at the *cis* face of the peroxisomal membrane, forming the receptor-docking complex. The import mechanisms show plasticity between kingdoms. In fungi, PTS1 and PTS2 import pathways are independent of one another until the docking stage (figure 4).

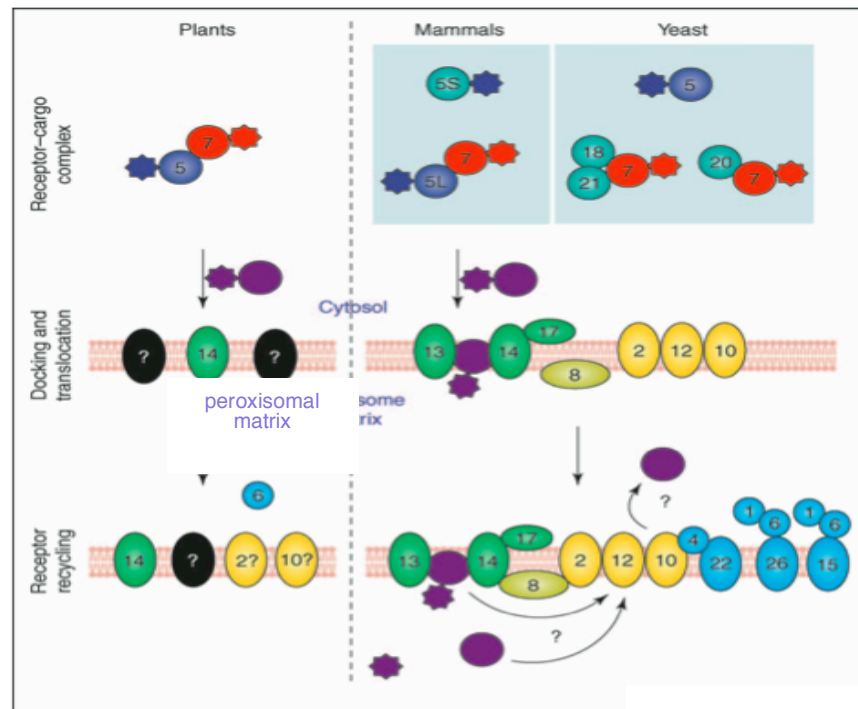


Figure 4. Import of peroxisomal matrix proteins. Receptor-cargo complexes composed of PTS1 cargo proteins (blue star) or PTS2 (red star) bind to pex5p or pex7p receptors respectively. The pex7p-cargo complexes requires accessory factors for import; in plants and mammals it binds pex5p, in *S cerevisiae* it binds pex18p and pex21p and in *Neurospora* it binds pex20p. Docking proteins (green) include pex13p, pex14p, and pex17p. In *S. cerevisiae* pex8p has been shown to bridge two complexes that are involved in docking (pex13p, pex14p, and pex17p; in green) or translocation (pex2p, pex10p, pex12p; in yellow) in yeast and mammals. Additional protein components involved in events downstream of receptor-cargo translocation (pex4p, pex22p, pex26p, pex15p, pex1p and pex6p) are shown in blue. (adapted from Baker and Sparkes 2005 review)

In mammals, splice variants of pex5p result in long and short variants (Pex5S and pex5L) both of which bind to PTS1 containing proteins. Pex5L also binds the PTS2- pex7 complex allowing PTS2 mediated import (42). Following receptor-protein complex docking in mammalian cells, trans-membrane import is mediated via the RING finger domains of pex12p, pex10p and pex 2p. Currently, it is hypothesized that the three RING finger peroxins function in concert in the translocation of receptor-cargo complex across the peroxisomal membrane (43). Pex8p has been described as a central organizer of the core import complex or importomer (44). It was shown that the docking complex and the RING complex are associated into a larger import complex by the action of pex8p on the trans side of the peroxisomal membrane (44). In *Pichia pastoris*, pex8p (PpPex8p) PpPex8p itself enters the matrix via dual import pathways, PTS1 and PTS2 in a pex14p dependent manner (44). It is thought that pex8p evolved redundant pathways as a means for efficient import of the protein or as a backup mechanism (44).

Import of the receptor-cargo complex into the peroxisome is followed by dissociation and export of the receptor back into the cytosol for further rounds of import (42). Export of pex5p and possibly pex7p occurs by an ATP-dependent process involving interaction with pex1p and pex6p members of the AAA family of ATPases. Pex1p and pex 6p are anchored to the membrane by interaction with pex15p (*S. cerevisiae*) or pex26p (humans). It has been demonstrated that pex5p becomes ubiquitinated during import (45,46,47). It is hypothesized that this ubiquitination of pex5p serves as a signal for export

back to the cytosol (48). Recently, the ubiquitin dependent recycling of pex20p in *P. pastoris* has been shown (49). Importantly, strong mechanistic and sequence similarities exist between pex20p and pex5p thus implicating a role for ubiquitination in the recycling of both peroxins (49).

Proliferation of peroxisomes

Proliferation and degradation is regulated in response to environment signals. Proliferation of peroxisomes in eukaryotes occurs via three major pathways . In constitutively dividing cells, they divide by fission of pre-existing peroxisomes . In this manner, peroxisome division and the number of peroxisomes is regulated in a geometric fashion. A second process, termed peroxisome proliferation, results in the rapid increase in peroxisome number. Lastly, peroxisome number is controlled by pexophagy a process that refers to the specific turnover of peroxisomes by autophagy (50)

Division of peroxisomes is dependent upon dynamin-related proteins (DRPs) , a class of GTPases that are also involved in the division of mitochondria and chloroplasts (51,52) Evidence from observation of tubular membranes formed in DRP mutant cells suggest that DRP acts at a late stage in peroxisome division to pinch off small peroxisomes from constricted tubules (51).

Recently, several PEX genes with roles in peroxisome proliferation and division have been identified. PEX11, initially cloned in *S. cerevisiae* was shown to function as a homo-oligomer affecting the size and number of peroxisomes.

Other members of this family have been identified based upon homology to PEX11. These members, which include PEX25 and PEX27 which also function as homo-oligomers, a characteristic of this family. Additional PEX genes with roles managing separation of peroxisomes have been identified including PEX28 and PEX29 in *S. cerevisiae* (51). Additional PEX genes in *S. cerevisiae* including PEX30, PEX31, PEX32 have been shown to control peroxisome size and number (51).

Certain signaling events in *Y. lipolytica* have been shown to play a role in proliferation of peroxisomes. Pex16p and acyl-CoA oxidase (Aox) complex interaction controls and regulates the proper division of mature peroxisomes (51).

In humans, peroxisome proliferation is controlled at the transcriptional level by activation of peroxisome proliferators activated receptors PPAR α and PPAR γ . PPARs are part of the nuclear hormone receptor superfamily of transcription factors. PPARs form heterodimers with another nuclear hormone receptor, retinoid X receptor (RXR) and bind to target sequences known as peroxisome proliferators response elements (PPREs). Three subtypes of PPARs have been identified, α , γ , δ (or β). PPAR α is highly expressed in liver and regulates the expression of several genes involved in lipid metabolism. PPAR γ has two isoforms, γ 1 and γ 2 that are generated by alternative transcription start sites (53). PPAR γ 1 is expressed in many tissues including adipose tissue and immune cells (54) while PPAR γ 2 is expressed in adipose tissue and has a role in adipocyte differentiation (55).

PPAR β/δ is ubiquitously expressed and is involved in the physiology of certain tissues as well as lipid homeostasis (56).

Recently, it has been demonstrated that PPAR α and PPAR γ bind to the functional PPRE identified in PEX11 α (53). In mammals, PEX11 has been implicated in peroxisome proliferation by recruiting mammalian DRP, DLP1/Drp1, to the peroxisomal membrane (57). While the natural peroxisome proliferating signals in mammals are not fully characterized, certain growth factors and polyunsaturated fatty acids (PUFAs) have been shown to cause peroxisome proliferation and tubulation in HepG2 cells (58).

Isoprenoid dysregulation in peroxin deficient mice

In studies of mouse models lacking functional peroxisomes, results indicate profound disturbances in the isoprenoid biosynthetic pathway. Recent studies in PEX2 $^{-/-}$ mouse liver demonstrated profound upregulation of HMGCR, IDI and FPPS activity in liver (59). Moreover, total cholesterol in the plasma and liver of these mice was reduced 43% and 40% respectively. These data further illustrate the role of peroxisomes in the regulation and homeostasis of the isoprenoid pathway.

Isopentenyl diphosphate isomerase (IDI): A key role in isoprenoid biosynthesis

The enzyme isopentenyl diphosphate isomerase (IDI; EC 5.3.3.2) plays a central role in the isoprenoid biosynthetic pathway. IDI transforms unreactive IPP into its reactive isomer DMAPP by the concerted addition and abstraction of protons at C-4.

These two isomers are the building blocks for the successive head-to-tail condensation reactions that result in the synthesis of C₁₀ GPP, C₁₅ FPP, and ultimately, non-sterol products and cholesterol (60).

IDI first identified in *Saccharomyces cerevisiae*, IDI, was determined to be a monomer with a molecular weight of approximately 40 kD (61). The yeast enzyme demonstrates a broad pH range with a K_m^{IPP} of 35 μM . Several lines of evidence indicated post-translational modification of the protein including prediction of several glycosylation sites (61). Using a plasmid shuffle technique, a cDNA clone from *S. pombe* was identified (61). The 227 amino acid *S.pombe* enzyme shows a high level of similarity with the homolog in *cerevisiae*.

IDI has been purified from *E coli*. The enzyme shows a K_m^{IPP} of 5 μM and has a requirement for the Mn^{2+} and Mg^{2+} for activity. It was shown that in the *E coli* enzyme, Mn^{2+} conferred greater activity at lower concentrations than Mg^{2+} , but both cations conferred equal activities at higher concentrations. IDI has been identified and characterized in numerous mammals including *sus domesticus* where the enzyme demonstrated K_m^{IPP} of 2.7 μM at pH 6.3 (61). The porcine enzyme showed a requirement for both Mn^{2+} and Mg^{2+} at concentrations ranging from 2-8 mM. ATP strongly inhibited the enzyme at concentrations of 1-5 mM. In contrast, the enzyme activity was enhanced by addition of mevalonate (1 mM) as well as dithiothreitol and mercaptoethanol at concentrations of 5 mM. Interestingly, two isoforms of IDI

were identified in this study based upon their differences in retention on DEAE-cellulose. Further analysis indicated that the isozymes demonstrated differences in pI values.

Multiple IDI isoforms

Four different isoforms of IDI have been isolated from avian liver (62) The isoforms demonstrated similar pH optima and their activities were dependent upon Mn^{2+} . The isoforms showed different K_m values (1.7, 1.5, 1.3 and 2.5 μM respectively).

In plants, the various isoforms have also been identified in different compartments, related to specific functions (63) However, two cDNAs encoding two distinct IDIs were recently identified in the green unicellular alga *Haematococcus* (64). The conclusion is made that one form is involved with the carotenoid accumulation in the cytoplasm, suggesting that the different gene products may reflect a requirement for this enzyme in different cell compartments.

Human IDI

The human cDNA, cloned from human promyelocytes, contained a 684-bp open reading frame. The human enzyme was overexpressed in *E coli* and purified to greater than 90% homogeneity. The purified enzyme showed maximal activity of 4.1 $\mu mol\ min^{-1}\ mg^{-1}$ at pH 7.0 in the presence of Mg^{2+} , with a K_m^{IPP} of 33 μM (61)

IDI has been characterized from various prokaryotic and eukaryotic sources. Several instances of multiple isozymes exist. The role of either Mn^{2+} or Mg^{2+} is clear but there is considerable kinetic variation across species.

IDI catalytic mechanism and active site

Several studies have provided support for the protonation-deprotonation mechanism of isomerization for IDI (figure 5).

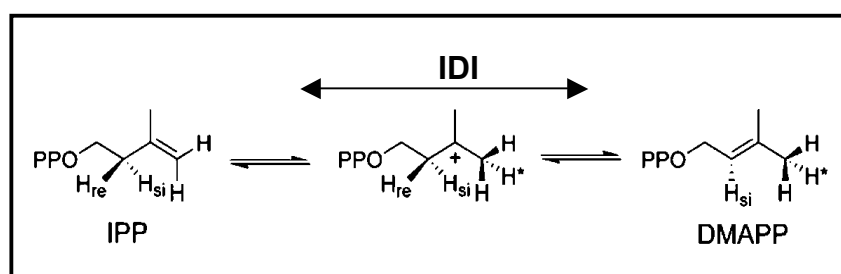


Figure 5. The mechanism of isomerization catalyzed by IDI. The reaction involves the antarafacial transposition of hydrogen and a carbocation intermediate. (adapted from Durbecq et al 2002)

These studies include proton exchange experiments (65), binding of transition state analogues and irreversible inhibition by mechanism based inhibitors (66). Inhibitor studies implicated two critical residues C67 and E116 (*E coli* numbering) in the proton transfer steps. Structural analysis determined by x-ray structure of the *E coli* enzyme indicated the antarafacial location of these residues (67). Studies indicated that E116 was involved in coordination of the divalent metal within the binding site.

Isolation of the enzyme with a transition state analogues identified a tertiary carbocation intermediate in which the C(3) is replaced by a ammonium moiety.

Analysis of the structure determined that E116 is coordinated through a carboxylate oxygen to the divalent metal and the H-N⁺ unit. In another structure the enzyme was inactivated with an epoxide derivative of IPP, the resultant oxirane ring was opened to yield a primary alcohol at C(4) with concomitant formation of a thioether bond between C(3) of the inhibitor and the sulfhydryl moiety of C67 (65). Further studies implicated the C67 residue as involved in the coordination of a second divalent metal (65). Site directed mutagenesis of the active site residues indicated that replacement of cysteine in the yeast enzyme by serine resulted in a modest 2-fold increase in K_m and a reduction in k_{cat} by $\sim 10^4$ (65). Importantly, replacement by alanine or valine resulted in an inactive enzyme. Similarly, substitution of the active-site glutamate with glutamine or valine completely abrogated activity. Recently, the primary role for the E116 residue has been elucidated in studies of a C67A mutant of E coli enzyme treated with an epoxy analogue of IPP. In this study, the oxirane ring was opened, forming an ester linkage between C(3) of the inhibitor and the side chain carboxylate of E116. Importantly, this study demonstrated that the inactive enzyme retained the ability to activate and open the oxirane ring, suggesting a role for E116 in the protonation step of the isomerization reaction (67).

IDI: a role in regulation of DMAPP-IPP pools

The possibility that IDI was involved in regulation of metabolic pools of IPP or DMAPP was investigated in several early studies. Metabolic pools of

DMAPP have been detected in plants via the existence of non-equivalent isotopic labeling of the IPP and DMAPP moiety of certain terpenoids (68, 69). In contrast, studies in certain plant species including *Mentha piperita* indicated preferential labeling of IPP moieties in mono and sesquiterpenoids following addition of labeled MVA (70). Thus, a regulatory role for IDI in the maintenance of C5 metabolic pools remains in question.

Regulation of IDI

In studies analyzing proteome changes induced in rat liver following treatment with the HMGCR inhibitor lovastatin researchers demonstrated a significant upregulation in the expression of IDI (71). Recently, a co-regulation of IDI and cholesterol biosynthetic genes in rat has been demonstrated (72). In this study, liver homogenates of rats fed a diet supplemented with cholestyramine and mevinolin, compounds shown to upregulate cholesterol biosynthetic genes through inhibition of HMGCR, showed a marked up-regulation of IDI mRNA and protein (72).

Similar results in liver from transgenic rats overexpressing SREBP1a or SREBP2 indicated that IDI mRNA was significantly upregulated in the livers of these animals (73). Further evidence is provided by sequence analysis of rat IDI indicating the presence of an SRE like region within the promoter (73). Taken together, these data provide strong evidence that IDI, as well as the majority of the genes involved in cholesterol biosynthesis, are regulated at the level of SREBP1a and SREBP2.

It has been hypothesized that compartmentalization and the existence of multiple isozymes of IDI provides a regulatory mechanism in plants and algae. In *Nicotiana tabacum* the two IDI isoforms are regulated at the transcriptional level under a variety of environmental conditions (74). Similar duplications exist in *Cinchona robusta* and the green alga *Haematococcus pluvialis* in both cases the two isozymes maintain specialized subfunctions by different expression patterns and subcellular localizations (63,64). It has been shown that, for many of the enzymes involved in isoprenoid biosynthesis, multiple isozymes are known.

Subcellular localization of IDI

As mentioned previously, a great deal of evidence has accumulated for the peroxisomal localization of enzymes involved in isoprenoid biosynthesis. Recently, IDI has been identified in hamster and rat where it was shown to localize to the peroxisome by a Pex-5p dependent PTS1 mechanism (72). In double-label immunofluorescence experiments, human fibroblasts and CHO cells were transiently transfected with HA-tagged IDI from hamster. The cells were immunolabeled with anti-HA and antibodies to the peroxisomal marker enzyme catalase. The punctate pattern obtained with IDI was superimposable with catalase (72).

Non-mevalonate pathway and IDI-type II

Recently, a mevalonate independent pathway leading to the synthesis of IPP and DMAPP has been identified (figure 6). The methylerythritol phosphate (MEP) pathway begins with the condensation of pyruvate with glyceraldehydes 3-phosphate (75, 76). The final reaction step yields not only IPP but DMAPP from 4-hydroxy-dimethylallyl diphosphate (77). Therefore, IDI is not essential or is absent in organisms that utilized the MEP pathway (78) Recently, it has been reported however, that in organisms including both the MEP pathway and the gene for IDI , disruption of IDI affected the phenotype (79) As a result, it has been proposed that in organisms in which IDI coexists with the MEP pathway IDI may serve to balance pools of IPP and DMAPP (80).

In addition to the previously characterized IDI another distinct form type II IDI has been recently identified (76). Type II IDI is found in some gram-positive bacteria (81), cyanobacteria (80) and achaea (82). Interestingly, type I and type II IDI have no sequence homology and different structures as well as different cofactor requirements. Type II IDI is a flavoprotein characterized by a TIM barrel. The enzyme requires FMN, NAD(P)H as well as a divalent metal for activity (83; 84). In contrast, type I is a compact globular $\alpha\beta$ protein requiring only divalent metal cations as cofactors (V Dubercq 2001). Type II IDI is proposed to proceed via a radical transfer reaction (86) while type I isomerase proceeds through an antarafacial protonation-deprotonation mechanism (87).

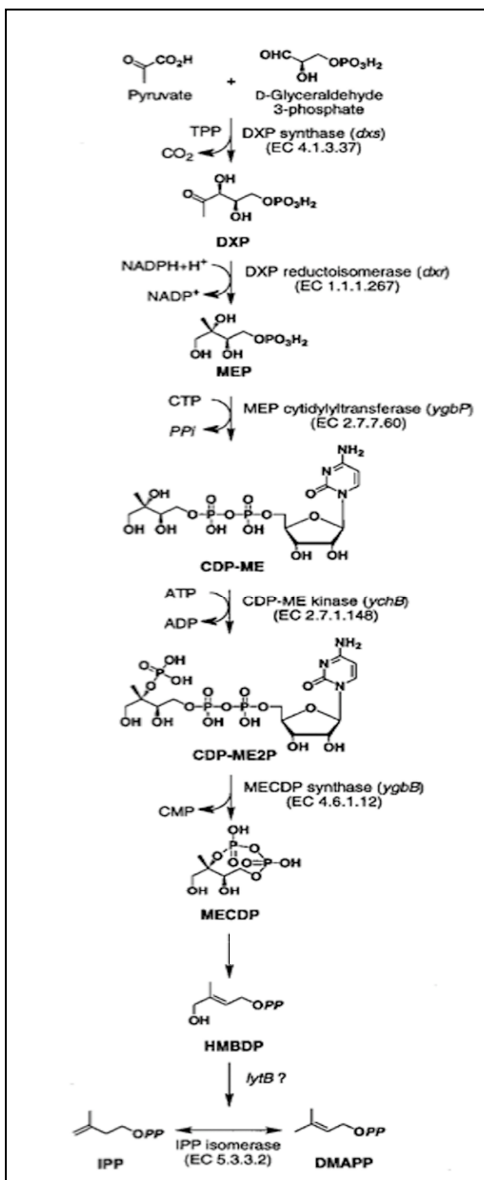


Figure 6. The non mevalonate or MEP pathway for synthesis of IPP and DMAPP (adapted from Kuzuyama 2002)

Identification of a novel IDI isozyme in mammals: IDI2

Recently, a novel muscle specific member of the isoprenoid biosynthetic family has been identified in mouse and human. We previously described this novel isoform of IDI1, designated as IDI2, and revealed that the isozyme lies as a tandem duplication of IDI1 in the genome of both mouse and humans (88). The tandem gene arrangement, conserved gene structure and high degree of similarity at the amino acid level suggest that the two genes arose by a recent duplication. Comprehensive phylogenetic analysis of known IDI enzymes led to a data set used to construct a highly resolved tree based upon maximum parsimony (88)(figure 7). The resultant phylogenetic tree reveals that the duplication of the IDI genes occurred before the divergence of mammals. This early date is corroborated by a different pattern of Alu repeats within the two genes indicating the duplication preceded the expansion of the Alu family 50-30 MYA.

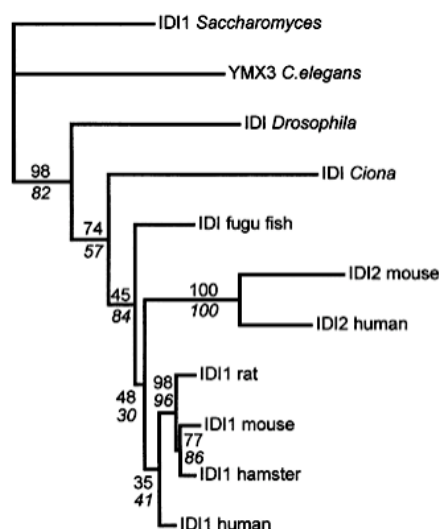


Figure 7. Phylogeny of IDI proteins in animals. The tree was reconstructed by neighbor-joining analysis of IDI protein sequences, gaps removed, and rooted with *S. cerevisiae* IDI. Bootstrap support in 100 pseudoreplicates is indicated at the branches (upper number = maximum parsimony; lower number = neighbor joining). Adapted from Breitling et al 2002.

It was shown that IDI2 is not a pseudogene but is rather a xaptogene under strong purifying selection. Pairwise alignments demonstrate that only translated regions of IDI2 show detectable similarity to the IDI1 gene, while introns and untranslated exons have diverged and retain no discernable similarity (figure 8). This pattern of sequence conservation implies that the mutations were not fixed randomly. Moreover, because the duplication occurred before the Alu expansion the distribution of Alu repeats in the IDI1 and IDI2 genes is independent thus IDI2 has survived a massive onslaught of Alu integrations and remained intact (88).

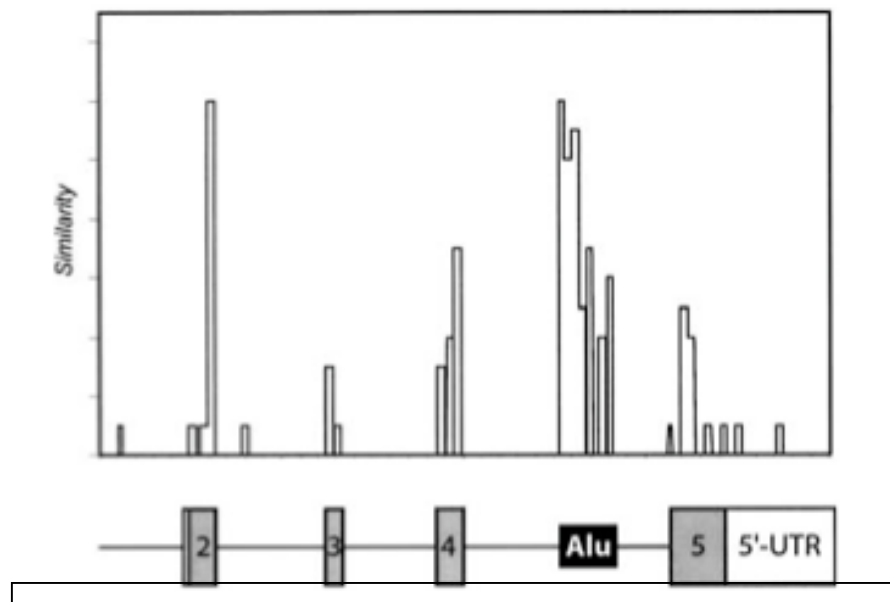


Figure 8. Conservation of the nucleotide sequence of human IDI1 and IDI2. A pairwise comparison (DiAlign) is shown in arbitrary units. Importantly, only the translated regions of the two genes show any measurable similarity. Numerous Alu elements have been incorporated independently into both genes after the duplication and their similarity is not the result of specialized selection pressure but rather due to a recent common ancestry. (adapted from Breitling et al 2002)

Analysis of the distribution amino acid differences between the two proteins on a structural model indicates that they are evenly distributed over the whole protein (figure 9). Thus it is unlikely that IDI2 has evolved a different substrate specificity from IDI1 (88).

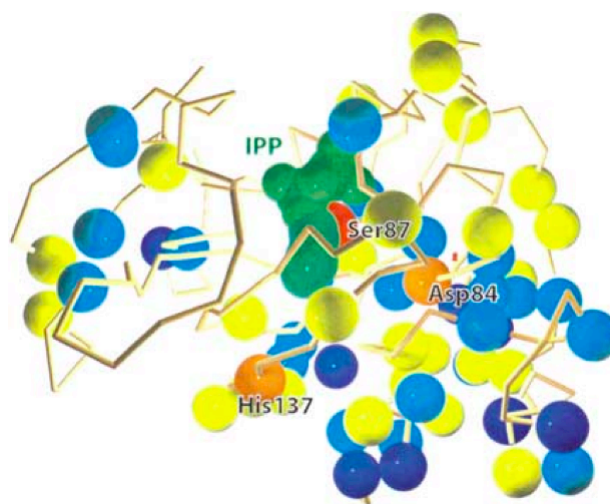


Figure 9. Structural model of IDI2 illustrating the distribution of residue differences between human IDI1 and IDI2. The residue differences are denoted by color according to measure of change. Amino acid changes of varying severity are found all over the protein and are not concentrated in specific regions. Red, Cys86Ser change within the putative active site; orange, Asn84Asp and Try137His changes in highly conserved residues; yellow, changes resulting in differences in hydrophobicity or charge in moderately conserved positions; blue, changes resulting in differences in charge or hydrophobicity in hypervariable sites; cyan conservative changes having no effect on charge and/or hydrophobicity; green isopentenyl diphosphate. Only the C-alpha atom is shown for each residue. (Adapted from Breitling et al 2002)

In addition, based on structural analysis IDI2 is likely to perform functionally as an isomerase despite the absence of one of the important catalytic residues (C86S). Based upon sequence similarity a model for the catalytic conversion of IPP to DMAPP was proposed (figure 10).

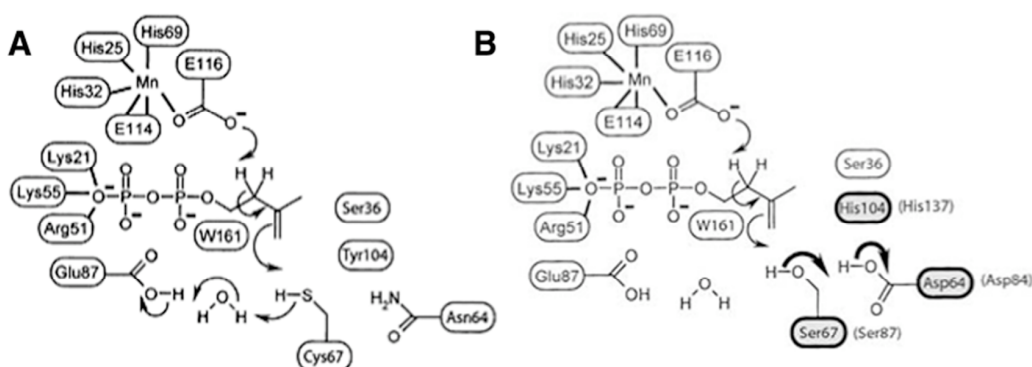


Figure 10. Model for isomerization reaction by IDI2. **A**, Proposed reaction mechanism of IDI1 involving a deprotonation/reprotonation of IPP by cysteine 67 and glutamate 116, respectively (modified after Durbecq et al. 2001) **B**, Model for possible reaction mechanism for IDI2. Exchange of asparagines 64 by aspartate can sufficiently increase the acidity of serine 67 to enable protonation of IPP. E coli numbering of amino acids is used for ease of comparison. (adapted from Breitling et al 2002)

EST data (dBEST) indicates that IDI2 is expressed exclusively in skeletal muscle in both human and mouse. This tissue specificity implies that IDI2 may play a muscle specific role in the isoprenoid pathway .

Role of Isoprenoids in skeletal muscle

Skeletal muscle, due to its mass and total energy requirement is the primary tissue responsible for the clearance of dietary lipids from the circulation and as a result plays a key role in maintaining overall metabolic homeostasis (89). Isoprenoids are involved at many levels in the organization of metabolic control in skeletal muscle. The role for small guanosine triphosphate (GTP)-binding proteins (G proteins) in skeletal muscle has been shown in regulation of the actin cytoskeleton, cell polarity, microtubule dynamics, membrane transport pathways, transcription factor activity and,

most recently, myogenesis (90). Isoprenylation of G-proteins is necessary for activation, mediated by formation of posttranslational lipid attachments (91).

Isoprenoids are also involved in the maturation of lamins, main components of the intermediate filament lamina of the inner nuclear membrane. In skeletal muscle, prelamin A undergoes a farnesylation-dependent proteolysis to become mature lamin A (91). Moreover, the importance of isoprenoid mediated activation in skeletal muscle is evidenced by the lethal skeletal myopathies that result from defects in lamin A processing implicated in Emery-Dreifuss muscular dystrophy (91).

In addition, a skeletal muscle requirement for isoprenoids has been shown in the the synthesis of selenoproteins. Selenoproteins play a key role in myogenesis and myoregeneration (91). Functional selenoproteins are formed by the enzymatic isopentenylation of selenocysteine-tRNA^{(ser)sec} (sec-tRNA) at adenosine 37. Isopentenylation is catalyzed by tRNA isopentenyl transferase, which uses IPP as a substrate. Notably, selenium deficiencies or defects in selenoproteins result in muscle myopathy (92)

Isoprenoids are also involved in the N-linked glycosylation of several proteins such as dystroglycan and IGF-1 which play a role in formation and maintenance of the cytoskeleton (91). N-linked glycosylation is a co-translational covalent modification involving terminally saturated dolichols, derivatives of both FPP and IPP.

Clinical Applications affecting Isoprenoids: Statins

Statins are a class of drugs that are potent inhibitors of cholesterol biosynthesis. Statins bind to HMGCR at nanomolar concentrations, leading to competitive displacement of the natural substrate HMG-CoA. In addition, inhibition of cholesterol biosynthesis is accompanied by an increase in hepatic LDL receptor, which promotes uptake and clearance of cholesterol from the bloodstream. While all statins inhibit hepatic HMGCR to varying degrees, important structural differences exist among the statins that distinguish their lipophilicity, half-life, and potency (93).

It has been estimated that 5 percent of individuals receiving statin therapy experience a debilitating muscle-related side effect. The myopathic reactions range from myalgia, elevated creatine kinase to the most severe case, rhabdomyolysis. The pathophysiology of statin-mediated muscle dysfunction remains poorly defined. The involvement of isoprenoids has recently been shown in the impairment of protein isoprenylation including RhoA and Rac1 resulting in alterations in actin cytoskeleton and assembly of focal adhesions (93) Additional experiments have shown that statin induced myotoxicity is specifically related to the inhibition of GPP but not through suppression of ubiquinone (94). These results suggested that myotoxicity was a result of reduced GPP prenylation of proteins leading to myotube apoptosis.

The molecular effects of statins on the isoprenoid pathway in skeletal muscle remains largely uncharacterized.

However, recent studies using microarray analysis in mouse and human muscle cells indicate that skeletal muscle is 5.5 to 44.5 times more sensitive to the effects of statins compared to hepatocytes (95).

Fibrates

Fibrates, another class of hypolipidemic drugs also function through inhibition of HMGCR thereby decreasing cholesterol synthesis.

Mechanistically, fibrates function through activation of PPAR α . PPAR α mediates transcriptional control of nuclear genes encoding proteins involved in fuel oxidation, including CPT1, pyruvate dehydrogenase kinase 4 (PDK4) and uncoupling protein 3 (UCP3). PPAR α null mice exhibit decreased expression of lipid regulatory genes, decreased β -oxidation and greater lipid accumulation in hepatic and cardiac tissue (96). Recent studies indicate that fibrates inhibit dolichol phosphate synthesis as evidenced in a decrease in isoprene chain length by an, as yet, unknown mechanism (97).

Dietary effects on isoprenoids in muscle

Skeletal muscle possesses a remarkable ability to adapt to changes in metabolic demand. In experiments using an RT-PCR based technique for performing nuclear run-on analysis, gene specific and fiber type specific changes in transcription rate following fasting were measured. In these studies it was found that a number of metabolic genes involved in and or required for lipid metabolism were increased (98). Interestingly, these increases were attenuated by the increased metabolic demand of endurance exercise.

Similarly it has been shown that expression of UCP3 in rats is enhanced following high fat feeding and is thought to be related to an increased need to handle lipids as a fuel substrate under this condition (99) This upregulation of lipid metabolism is controversial, however, as experiments analyzing muscle specific lipid metabolism gene profiles following either high fat or high cholesterol diets indicated that fatty acid translocase and β hydroxyacyl-coA dehydrogenase were increased significantly while expression of UCP3 was unchanged (100). Overall the dietary and drug induced effects on the isoprenoid biosynthetic pathway in skeletal muscle are largely uncharacterized. However this area is the focus of many recent studies particularly those involving the statin class of cholesterol lowering drugs.

Project goals

Our hypothesis is that IDI2 is a muscle specific isoform of IDI with the ability to catalyze the isomerization of IPP to DMAPP. As a result, IDI2 plays a specialized role in the isoprenoid biosynthetic pathway. Specifically, our objectives are to:

- i) determine whether IDI2 has functional isomerase activity and the ability to catalyze the conversion of IPP to DMAPP
- ii) determine the effect of a C86S mutation in human IDI1, as well as an S86C mutation in IDI2
- iii) examine the enzyme kinetics of IDI2
- iv) identify the subcellular localization of human IDI2
- v) investigate the role of IDI2 in muscle

The text of Chapter One, Introduction, in part, has been submitted for publication. The dissertation author was the primary researcher and author. The co-authors listed in this publication participated in the research which forms the basis for this chapter.

Characterization of IDI2, a Second Isopentenyl Diphosphate Isomerase in Mammals. Manuscript submitted. JBC. Clizbe D, Owens M, Shackelford J, Krisans SK.

Experimental Procedures

Cloning of human IDI2 (hIDI2)

A fragment of human IDI1 cDNA sequence was used to search the human subset of dEST at NCBI (<http://www.ncbi.nlm.nih.gov>). A clone, which revealed similarity to the 5'end of the IDI1 cDNA, was ordered from I.M.A.G.E. Consortium [LLNL] cDNA Clones (the I.M.A.G.E. Consortium: An Integrated Molecular Analysis of Genomes and their Expression," Lennon, G.G., Auffray, C., Polymeropoulos, M., and Soares, M.B. [1996] Genomics 33, pgs. 151-152) and sequenced as described (24)

Genomic structure

This work was generously assisted by Dr Adamski , Institute of Experimental Genetics, GSF-National Research Center for Environment and Health, Neuherberg, Germany. A genomic PAC library (female RPC16709) with four-fold coverage and an average insert length of 130 kb was obtained from the Resource Center, Primary Database of the German Human Genome Project at the Max-Planck-Institute for Molecular Genetics (Berlin,Germany) The library was screened with a randomly primed [α -³²P]-dCTP labeled probe corresponding to the full length of the human IDI1 or IDI2 coding sequences. The probes for IDI1 and IDI2 recognized the same eight clones which were confirmed by PCR. The primers were derived from the most 3' sequences of cDNA for IDI1 or IDI2 which were assumed to rest in one exon.

The following primer pairs were used: forward 5'-CAGAATTACAGTGAGCACAGAGGTG-3' and reverse 5'-CTGACTCTGTAGACAGTGGTCCTC-3'.

Northern blot analysis

Human multiple tissue Northern blots were purchased from Clontech. Each blot contained approximately 2 µg of poly(A)⁺RNA. Hybridizations were performed with [α -³²P] - dCTP labeled probes. Total RNA was isolated as described by Chomczynski and Sacchi. Poly(A)⁺ RNA was isolated from total RNA using Oligo (dT)-cellulose (Roche) according to the supplier's protocol. Poly(A)⁺ RNA was electrophoresed in a 1.0% MOPS buffered agarose gel under denaturing conditions for one hour at 100 V. The RNA was then transferred to a nylon membrane overnight using the Posiblot Pressure Blotter and Pressure Control Station (Stratagene, San Diego, CA) as described by the manufacturer's protocol. The blot was UV cross-linked using the Stratagene Crosslinker. Unincorporated nucleotides were removed from the labeled DNA by the NucTrap Isolation system from Stratagene. The [α -³²P]-dCTP labeled probes consisted of the 687 bp IDI-1 and 684 bp IDI-2 open reading frames. After prehybridization for 2 h, blots were hybridized overnight at 42 °C with the labeled probes in a solution of 50% formamide, 6% SDS, 100mM Na₂PO₄, and 200mM NaCl. Low stringency washes of the probed membranes consisted of one wash for 15 minutes at 42 °C with 2x SSC and 0.1% SDS. Following the washes the membranes were exposed to a

Phosphorimager screen (Molecular Dynamics). Quantification was performed using Molecular Dynamic's ImageQuant software.

Primate active site analysis

Primate genomic DNA was generously donated by Dr. Oliver Ryder (Centre for Reproduction of Endangered Species San Diego Zoo San Diego CA 92112-0551). Oligonucleotide primer pairs were designed from human IDI1 and IDI2 to flank exon 3 containing the active site region. IDI2 Forward 5'TGCATTTTTTCCAGGGTATTTTACCGACTCC3' Reverse 5'TTGTCATGAACACAATGTCCTCTGG3'; IDI1 Forward 5'GTTGTTTTACGAATACGTGT3' Reverse 5'CAGACTGAGCTTTGTAGTGAATTCG3'. The loci were screened by PCR amplification of primate genomic DNA. PCR mixtures consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, each primer at 0.2 µM, a 0.2 mM concentration of each deoxynucleotide triphosphate, 10% DMSO, 50 ng of genomic DNA, and 2.5 U of *Taq* DNA polymerase in a final volume of 30 µl. PCR amplification was performed with a Perkin Elmer Thermalcycler System 9700 thermal cycler. PCR was performed by starting with an initial denaturation step of 3 min at 95°C, followed by 30 cycles each of 30 s at 95°C, 30 s at 58°C, and 60 s at 72°C. The amplification products were resolved by electrophoresis through 1.5% (wt/vol) MetaPhor (FMC Bioproducts, Rockland, Maine) agarose gels (15 cm by 15 cm) at 3 V/cm. Ethidium bromide-stained gels were photographed and visually inspected for expected size. Positive amplicons were excised from agarose gel and purified

(Qiaquick gel extraction kit, Qiagen). Products were cloned into pCR-Blunt II-TOPO (Invitrogen) by manufacturers protocol. Recombinant clones were amplified in TOP10 competent cells (Invitrogen). The IDI1 and IDI2 primate sequences were verified in triplicate by DNA sequencing using vector flanking sequences (MicroCoreFacility, San Diego State University, San Diego, Ca).

Cell Culture and transfection conditions

HeLa cells were maintained in Eagles Minimum Essential Medium (EMEM) (Gibco) containing 10% fetal bovine serum (FBS;LifeTechnologies), penicillin (100 units/ ml), and streptomycin sulfate (100 µg/ml) at 37° under 5% CO₂. The mouse myoblast cell line C₂C₁₂ (American Tissue Culture Collection, Rockville, MD) was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco® - Invitrogen, Carlsbad, CA) supplemented with 1mM sodium pyruvate, 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO), fungizone (1.8 µg/mL), penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were grown at 37°C in a 5% CO₂ environment. Cells were maintained at ≥ 50% confluency. For growth in LPDS, cells were incubated in DMEM supplemented with 1mM sodium pyruvate, 5% lipoprotein deficient serum (LPDS, Intracel, Frederick, MD) without antibiotics for 24 hours. Cells were transected using Lipofectamine-2000 (Invitrogen). All transfections were performed under the recommended protocol provided by supplier. Cells were selected for in growth media plus 1 mg/mL G418. Stably transfected cell lines were maintained in growth media containing 0.5mg/mL G418 (Cellgro®),

Mediatec, Herndon, VA). Over-expression of IDI2 in C₂C₁₂ cells was verified by real-time quantitative RT-PCR.

RNA isolation

Cells for RNA isolation were harvested by trypsinization and pelleted by centrifugation at 1k RPM for 10 minutes at 4°C. The pellet was flash frozen in liquid nitrogen. RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA isolation from skeletal muscle was performed on flash frozen tissue with RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. RNA from liver was isolated with RNeasy Midi Kit (Qiagen) according to the manufacturer's protocol. DNase digestion was performed on the column using RNase free DNase I (Qiagen).

cDNA synthesis

cDNA synthesis was performed using SuperScript III (Invitrogen), Oligo(dT) primer (Invitrogen) and 5 µg total RNA according to the manufacturer's protocol. In the case of cDNA from human skeletal muscle, only 1 µg of total RNA was used. The transcription step of the protocol was run for 60 minutes at 50°C.

E. coli and Mammalian expression vectors

Human IDI1 and IDI2 cDNAs were amplified from human skeletal muscle (IDI2) and liver (IDI1) by reverse transcriptase PCR (RT-PCR) as described. cDNAs were subcloned into pCR-Blunt II-TOPO (Invitrogen) by manufacturers protocol. Restriction sites for expression vector construction were introduced

by the following primers using PCR conditions 94° 1 min, 55° 30 seconds 72°

1 min 30 seconds for 30 cycles. IDI 1 Forward

5'GATATGTCGACAATTCCACCACACTGGACTAGTGG 3' Reverse

5'CATTACCTCGAGATTCACATTCTG3' , IDI2 Forward

5'GGGCTGCAGGATCGGCACGAGGAG3' Reverse

5'CTCGAGGGCCTCTCACACTCTGTG3' .Amplification products were

directionally cloned into pET19, pET32 ,pET41, and pET43 using Sal1 and

Xho1 restriction sites (IDI1) or Pst1 and Xho1 (IDI2). For mammalian

expression construct, the same strategy was employed except the following

primers were used for amplification IDI-I Forward

5'CAGACATATGGTAATGATGC3'

Reverse 5'CATTACCTCGAGATTCACATTCTG3' or IDI-2 Forward

5'CATATGCAGCAGCTATGTCTGA3'

Reverse 5'CTCGAGGGCCTCTCACACTCTG3' .Amplification products were

directionally cloned into pcDNA3.1(-) using Nhe1 and Xba1 restriction sites.

Recombinant constructs were amplified in TOP10 competent cells

(Invitrogen). The Full length ORF and cDNA sequences were verified in

triplicate by DNA sequencing (MicroCoreFacility, San Diego State University,

San Diego, Ca).

Murine IDI1 and IDI2 cDNA was obtained by using SuperScript

(Invitrogen), oligo(dT) primer and 5 µg total RNA according to the

manufacturer's protocol. IDI2 was amplified from mouse neonate skeletal

muscle cDNA with the following primers: Forward

5'CGGCGCTAGCACCATGGGATTTCCAGGCAAGCAAACTCACCTT and reverse 3'CGGTCAAGATCACAGTCCATATATCTTGTCAGGCTCCAC.

Primers include restriction enzymes sites for NheI and XbaI so that PCR fragments can be easily directionally cloned. PCR parameters were as follows: 2 min. at 95°, 1 min. at 62°, 1.15 min. at 72° then 30 cycles of 30 sec. at 94°, 45 sec. at 62°, 1.15 min. at 72° then 5 min. at 72°. PCR reaction mix included 4 µL cDNA, 1X PCR buffer (Invitrogen), 10 µM dNTPs (Invitrogen) and 2.5 U Taq DNA Polymerase (Roche, Basel, Switzerland). PCR reaction product was run on a 1% agarose gel. The PCR fragment was excised from the gel and purified with Qiagen's QIAquick Gel Extraction Kit according to the manufacturer's protocol. Purified fragment was inserted into TOPO TA vector (Invitrogen) following manufacture's protocol. TOPO TA vector containing cloned IDI2 and pcDNA 3.1 (-) (Invitrogen) were digested with NheI and XbaI overnight at 37°C. Digests were run on 1% agarose gel. The IDI2 fragment and linearized pcDNA 3.1 (-) were excised and purified with QIAquick Gel Extraction Kit (Qiagen) following manufacture's protocol. Purified fragments were then ligated using 2 units T4 DNA ligase (Roche) and 10X ligation buffer to 1X in the refrigerator overnight. The ligation product was transfected in One Shot® TOP 10 Chemically Competent *E. coli* (Invitrogen). Recombinants were verified in triplicate by DNA sequencing.

Yeast expression vectors

The complete coding regions of IDI1, ID1C86S, IDI2, IDI2C86S were amplified using forward primer 5'-CAGACATATGGTAATGATGC-3' and

reverse primer 5'-CATTTACCTCGAGATTCACATTCTG-3' (IDI1, ID1C86S) or forward primer 5'-CATATGCAGCAGCTATGTCTGA-3' and reverse primer 5'-CTCGAGGGCCTCTCACACTCTG-3' (IDI2, ID2S86C). PCR conditions 94° 1 min, 55° 30 seconds 72° 1 min 30 seconds for 30 cycles. Amplification products were cloned into pYES2.1/V5-His-TOPO vector (Invitrogen). pYES2.1/V5-His-TOPO contains the *URA3* gene for selection in yeast and 2 μ origin for high-copy maintenance TOPO cloning reaction was prepared according to manufacturers recommendation using 1 μ l each of the amplification products. 3 μ l of each ligation reaction was used to transform One Shot Top 10 F' (Invitrogen) competent E coli. Colonies were selected on LB kanamycin (50 μ g/ml) and recombinant plasmids isolated using Qiagen Mini-prep Plasmid Isolation kit. Recombinant plasmids were screened by PCR with forward primers to the Gal1 region of pYES2.1/V5-His-TOPO (5'-AATSTSCCTCTATACTTTAACGTC-3') and reverse primers to the 3' end of either IDI1 or IDI2, 5'-CATTTACCTCGAGATTCACATTCTG-3' or 5'-CTCGAGGGCCTCTCACACTCTG-3'. Positive recombinants were verified by DNA sequencing in triplicate (MicroCoreFacility, San Diego State University, San Diego, Ca).

EGFP-fusion constructs

Full length coding regions for IDI1 and IDI2 were amplified by PCR using oligo nucleotide primers that introduced unique 5'BglII and 3'Kpn1 restriction sites, IDI1 forward 5'- CCCATAGATCTGATCAGACATTTTGTAAATGATG-3', IDI2 forward 5'-AGGTCGGGTCAGATCTAGCAGCTAT-3', IDI1 Reverse 5'-

GTAATCAGGTACCTACATATTCAC-3' IDI2 Reverse 5'-

CATTACACATGGCTGACTGGTACCTCCCTGGCCTCTCA-3'. PTS1 deletion constructs were created by amplifying full-coding region template with reverse primers that added a unique Kpn1 restriction site and a tga stop codon upstream of the final 3 amino acids IDI1 Δ PTS1rev 5'-

GTAATCAGGTACCTACATATTCACATTCTTCATATTTTCTC-3' and IDI2 Δ PTS1rev- 5'-TGGCTGACTGGTACCTCCCTGGCCTCTCACACTCTTCATAT-3'. The amplification products were subcloned into pCR4-TOPO (Invitrogen). The fragments were excised by restriction digest with BglII and Kpn1. The excised fragments were cloned into pEGFP-C3 (Clontech). The recombinant pEGFP constructs were then determined to be in frame with the N-terminal EGFP and the PTS1 deletions were confirmed in triplicate by DNA sequencing.

Active site mutagenesis

The entire ORF of IDI1 and IDI2 was amplified in a 2 step process. Using oligonucleotide primers to the 5' end that added an Nde2(IDI1) or Nde1(IDI2) restriction site (CAGACATATGGTAATGATGC, 5'CATATGCAGCAGCAGCTATGTCTGA3') and a internal reverse primer that added a unique Bsr1 (IDI1) or BssS1 (IDI2) restriction site (5'ATGACTGGAACACGTATTCGTAAAACA3',5'GTCACAACACGAGTCGGTAAATACCCAGG3') we amplified a 351 bp fragment (fragment A) which was gel purified and subcloned into pCR-Blunt II-TOPO vector (Invitrogen). We then amplified the remaining 350 bp (Mutagenic fragment B) fragment and

introduced the C-S (IDI1) or S-C (IDI2) amino acid change in the sequence using an internal forward primer containing a Bsr1 (IDI1) or BssS1 (IDI2) site (5'TGTTTTACGAATACGTGTTCCAGTCAT3', 5'TTTACCGACTCGTGTTGTAGCCACCCATTATAC3') that also introduced sequence to generate the desired mutation and a reverse primer complementary to the 3' end of the reading frame (5'CATTACCTCGAGATTCACATTCTG3', 5'CTCGAGGGCCTCTCACACTCTG3') containing a Xho1 restriction site. Fragment B was then gel purified and subcloned into pCR-Blunt II-TOPO vector. Fragment A for IDI1 and IDI2 were isolated by restriction digest with Nde1 and Bsr1, and Nde and BssS1. Fragment B for IDI1 and IDI2 was then isolated from pCR-Blunt II-TOPO by restriction digest with Bsr1 and Xho1, or BssS1 and Xho1. Fragments A and B were ligated using T4 ligase and cloned into pcDNA 3.1(-). Recombinant pcDNA3.1IDI1C86S and pcDNA3.1IDI2S86C were amplified in TOP10 competent cells (Invitrogen). The Full length ORF and sequence containing desired mutations were verified in triplicate by DNA sequencing.

SDS–PAGE and Western blotting analysis

SDS–PAGE was carried out on NuPAGE Novex Bis-Tris 1.0 mm gel in MES SDS Running Buffer (Invitrogen) under reducing conditions. Proteins were visualized by Coomassie Blue R250 staining. For Western blotting, the proteins were separated by NuPAGE Novex Bis-Tris 1.0 mm gel under reducing conditions and then electrotransferred onto a nitrocellulose membrane (Millipore) in NuPAGE transfer buffer using a XCell II Blot module

(Invitrogen). Non-specific protein binding was blocked by incubating the membrane in PBS containing 0.05% Tween 20 and 5% BSA (Sigma) at 4 °C overnight. After washing with PBS–Tween 20, the membrane was then incubated with either monoclonal mouse anti-His-6 conjugated with peroxidase (1:10,000) or rabbit anti-IDI1 or anti-IDI2 (1:50, 1:20) for 1 h at room temperature under constant shaking. The membrane was washed three times for 10 min with TBS–Tween 20 and incubated at 37 °C with the appropriate horseradish peroxidase proteinA conjugate (BioRad) for 1 h, and subsequently washed three times for 10 min each with PBS–Tween 20. Bound antibody was revealed by the addition of Western Lightning Chemiluminescence Reagent PLUS (PerkinElmer) substrate.

Antibody preparation

IDI1 and IDI2 antibodies were designed to recognize the peptide sequence DNLNHLSPFVDHEK (IDI1) and PHLEDVSSFVEPDK (IDI2) representing amino acids 210 to 223. These peptide sequences were submitted to Multiple Peptide Systems (3550 General Atomics Court, San Diego, CA 92121) for synthesis and production of rabbit polyclonal antisera. The antisera were evaluated for titer, and specificity by Western blot analysis against bacterially expressed human IDI1 or IDI2. Because the crude antisera showed cross reactivity with either IDI1 or IDI2 as well as various non-specific E coli proteins we affinity purified both antibodies as follows. Bacterially expressed hIDI1 and hIDI2 were coupled to CnBr-sepharose 4B (Pharmacia) by standard protocols.

Aliquots of anti-IDI2 sera were first passed over E.coli lysate–agarose (Pierce) and the flow through applied to a IDI1-sepharose column and rocked at room temperature for 2 hours. The flow-through from this column was then applied to an IDI2-sepharose column, rocked for 2 hours, and washed. Affinity purified antisera were then eluted from the column with 0.2M glycine. IDI1 antiserum was purified using the same procedure except that the loading order onto IDI1 and IDI2 coupled sepharose were reversed. Specificity and affinity was tested by Western blot analysis

Yeast transformation and expression

Diploid yeast strain InvSC1 was transformed by LiAc/TE/PEG method described by supplier (Invitrogen) using 1 µg each pYES2.1IDI1-V5-His, pYES2.1IDI1 C86S-V5-His, pYES2.1IDI2 -V5-His, or pYES2.1IDI2 S86C-V5-His. Transformants were selected on SC–ura plates. To screen positive transformants for recombinant plasmids colonies were resuspended in 20 µl of dH₂O. 1.0 µl of resuspension was used in a 50 µl PCR reaction with forward primers spanning the Gal1 region of pYES2.1 (5'-AATSTSCCTCTATACTTTAACGTC-3') and reverse primers to the 3' end of either IDI1 or IDI2, 5'-CATTTACCTCGAGATTCACATTCTG-3' or 5'-CTCGAGGGCCTCTCACACTCTG-3'. PCR conditions were 95° 5 min (1 cycle), 94° 30 sec, 55° 45 sec, 72° 1 min (35 cycles) 72° 5 min. Positive transformants were then used for expression studies. Expression of pYESIDI1-V5-His, pYESIDI1C86S-V5-His, pYESIDI2-V5-His, pYESIDI2S86C-V5-His was performed by methods described by manufacturer (Invitrogen).

Aliquots of induced cultures were removed at various time points (0, 4, 8, 12, 16, and 24 hours) post-induction. Cell lysates were prepared per recommended protocol. Cell lysates were used directly in enzyme assays or assayed for protein by Bradford method standardized with bovine serum albumin, and estimated by its molar extinction coefficient at 280 nm. It was noted that in cultures designed to over-express pYES IDI and pYES IDIC86S cell density was 40-50X lower in cell density (data not shown). Importantly, the ratio of expressed IDI per volume of total cell extract was unchanged in cultures with decreased expression times (4, 8, 10 hours) and increased culture volumes (25 ml, 50 ml, 100 ml) or induced with lower concentrations of galactose (5, 10, 20%). The possibility exists that the differences in IDI expression between samples is explained by increased proteolysis in IDI1 and IDI1C86S over-expressed cultures. However, addition of protease cocktail did not affect the overall yield of expressed protein. Given the apparent growth inhibition in IDI1 or IDI1C86S cultures it seems likely that the over-expression of IDI1 in a background of endogenous IDI1 expression leads to profound cell toxicity. Similar results have been shown in tissue culture when IDI1 was over-expressed in an IDI1 expressing cell line (data not shown).

hIDI1 and hIDI2 partial purification

50ml of IDI1 and IDI2 expressing yeast cultures were centrifuged at 5 k rpm to pellet. Pellets were washed 2X in 5mM Hepes, 10% glycerol pH 7.0, and processed immediately or frozen at -70°C . The remaining procedures

were performed at 4°C or on ice. Frozen pellets were thawed, rinsed with 5mM Hepes, 10% glycerol, 10mM DTT including protease inhibitors. (Roche Protease complete mini tablet) Acid washed glass beads (Sigma) were pre-rinsed with 5mM HEPES, 10% glycerol plus protease inhibitors in a Corex glass tube. Yeast pellet suspension was added and homogenized by repeated cycles of vortexing and chilling in an ice water bath (30 seconds each, five cycles). The homogenate was centrifuged (Bio-Rad, microfuge) 3 minutes at 4°C and the supernatant collected. The homogenation procedure was repeated for a total of three times. Supernatants were brought to 55% by addition of solid ultra-pure ammonium sulfate (ICN) Precipitating proteins were removed by centrifugation and the supernatant was brought to 75% ammonium sulfate. Precipitating proteins were collected, resuspended in buffer plus 0.5 mM benzamidine, 0.1mM Pefabloc, 100 µM leupeptin, 5 µg/ml pepstatin A, 1.28 µg/ml aprotinin and dialyzed overnight at 4°C. Samples were collected and diluted with 10% glycerol and applied to a DEAE cellulose column equilibrated in buffer only. Proteins binding were eluted with a 0-0.5M KPO₄ gradient in buffer without protease inhibitors. Fractions containing IDI activity were pooled, diluted with water and reappplied to a new DEAE column, and eluted with 0.5M KPO₄ to concentrate. The concentrated fractions were applied to a Sephacryl S-200 (Sigma) column in 5mM Hepes, 10% glycerol, 10mM DTT, and fractions containing IDI activity located by enzymatic assay.

Isomerase activity assay

Our assay is a modified version based on the acid lability of the allylic diphosphate DMAPP described by Satterwhite (11). The assay mixture consisted of 0.05 M Hepes buffer (pH 7.0), 20 mM MgCl_2 , 10 mM DTT, 10 μM [^{14}C]IPP (10 $\mu\text{Ci}/\mu\text{mol}$; American Radiolabeled Chemicals, St. Louis) and varying amounts of cell extract containing IPP to a final volume of 100 μl . The reaction was initiated by adding enzyme to the pre-warmed assay system followed by incubation at 37°C for 10 min. The reaction was terminated by adding 0.4 ml of 25% concentrated HCl in MeOH. 0.05ml of ^3H -FPP in 70% EtOH, 25 mM NH_4HCO_3 was added as a standard, followed by incubation at 37°C for 10 min. Each sample was extracted twice with 2.0 ml aliquots of petroleum ether. The extracts were combined and the radioactivity was determined in a scintillation solution (Ecolite) by using a scintillation counter (LS 6000LL; Beckman Coulter).

Plasmid shuffle

pYESIDI1 and pYESIDI2 were shuffled into haploid *S cerevisiae* IDI1 deletion strain FH2-5b (generous gift of Poulter lab, University of Utah) by methods described previously (12) with few modifications. pYESIDI1 and pYESIDI2 were transformed into competent FH2-5b by LiAc/TE/PEG method (Invitrogen). No pYESIDI1 double transformants were recovered. Following incubation at 30° C for 3 days on solid SG –ura-leu-lys media, pYES IDI2 double transformants were washed from plates in SG (20% galactose) –ura-

leu-lys media containing α -AA (3 mg/ml) transferred to α -AA-20%galactose (3mg/ml) solid media plates and incubated at 30° C for 72 hours. Surviving colonies were then replica plated on onto the same medium or solid α -AA medium containing glucose as sole carbon source. This method selects for those colonies able to survive when the shuffled gene is actively transcribed but perishes when transcription is repressed by glucose. By this method we were able to identify IDI2 true positive shuffle transformants.

Yeast colony PCR

Colonies shown to survive on α -AA medium including galactose but not on α -AA medium including glucose were resuspended in 20 μ l dH₂O. 1 μ l of a 1:10 dilution of these resuspensions were used in a 50 μ l PCR reaction using pYes 2.1Gal1 forward primer 5'-AATATACCTCTATACTTTAACGTC-3' and IDI2 reverse primer 5'-CACTCTGTGTATTTTGTGAAG-3' (pYes 2.1IDI2) or pRS317IDI1 forward 5'-GTAATACGACTCACTATAGG-3' and pRS317IDI1 reverse 5'-CCTCACTAAAGGGAACAAAAG-3'. PCR conditions were 95° 5 min (1 cycle), 94° 30 sec, 55° 45 sec, 72° 1 min (35 cycles) 72° 5 min. PCR products were analyzed by agarose gel electrophoresis.

Animals

Mice were male Black Swiss (Taconic), a cross between C57/BL6 and Swiss Webster. Mice were sacrificed at approximately 100 days of age, following the completion of experimental protocols. They were anesthetized

with a mix of CO₂ and O₂ prior to decapitation. Tissues were immediately harvested, flash frozen in liquid nitrogen and stored at -70°C.

Mouse diet

All diets were administered for 30 days, *ad libitum*. HFD was Harlan Teklad's special 1.25% cholesterol diet (TD96335). Statin diet was prepared with 0.1% Simvastatin (Sigma-Aldrich). Fibrate diet was prepared with 0.2% gemfibrozil (Sigma-Aldrich).

Exercise protocol

Mice were exercised for five weeks on a custom made motorized cylinder measuring 74mm in diameter(0.23m/rotation). Mice were exercised for 1 hr 5X week per previously described protocols(13,14,15). Mice were sacrificed within 24 hours of their final exercise period

Quantitative Real-Time PCR

The mRNA levels were quantitated using a Bio-Rad iCycler (Bio-Rad, Hercules, CA). iQ SYBR Green Supermix (Bio-Rad) was used for all experiments. Experiments were done on 96 well plates (Axygen, Union City, CA) with a 25 µl reaction volume. Primers were used at a concentration of 400 nmol per well except Mouse GAPDH 2, which was used at a concentration of 250 nM per well. Primer specificity was tested by running a gradient program with a positive control containing cDNA and a negative control containing water for 40 cycles at 95°C for 20 sec. and a temperature gradient of 55 – 65°C for 30 sec.

Each sample was run in triplicate and normalized to GAPDH. Experimental plates were run for 40 cycles at 95°C for 20 sec. and 55° for 30 sec.

Primer Name	Primer Sequence
Mouse IDI1, primer 1	F 5' TTCACCAATAGTTGCTGTAG3' R 5' ATCCATTTTCATTTAGATCAACC3'
Mouse IDI1, primer 2	F 5' GGTTTCAGCTTCTAGCGGAGA3' R 5' TCGCCTGGGTTACTTAATGG3'
Mouse IDI2	F 5' GATTGGCTACCTTCTGTTG3' R 5' CTGAACCAAGGGGTGATC3'
Mouse HMG-CoA reductase	F 5' CTTGTGGAATGCCTTGTGATTG3' R 5' AGCCGAAGCAGCACATGAT3'
Mouse GAPDH, primer 1	F 5' GCCTTCCGTGTTCTACCC3' R 5' TGCCTGCTTCACCACCTTC3'
Mouse GAPDH, primer 2	F 5' GTGTCCGTCGTGGATCTGA3' R 5' CAAGAAGGTGGTGAAGCAGG3'

Sample preparation for assays

Cells were plated at a density of 2×10^5 cells per 100 mm dish and harvested 48 hours later. For LPDS experiments, 24 hours after seeding the cells, the plates were rinsed three times in PBS and media was replaced by 5% LPDS media.

Plates were rinsed three times in CMF-PBS then cells were scraped in CMF-PBS into 1.5 ml tubes and pelleted at 1000 x g for 10 minutes at 4°C.

Supernatant was removed and pellet was resuspended in homogenization buffer. Cells were homogenized 20 strokes with a motor-driven homogenizer.

Homogenate was then centrifuged at 1000 x g for 10 minutes at 4°C.

Supernatant was removed and used for protein determination and enzyme activity determination. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

HMGCR assay

HMGCR activity in cells was determined as previously described (16), with the following modifications: Activity in the cellular extract was determined using 100 µg of protein, incubated for 1 hour at 37°C. Liver samples were homogenized in CMF-PBS plus 0.4% triton, 100 µM leupeptin, 100 µM pefabloc and aprotinin. Following homogenization, the assay followed the same methodology as above except that 100 µg of protein was incubated for 40 minutes at 37°C.

Cholesterol synthesis assay

Cholesterol, fatty acid and dolichol phosphate rate of synthesis was determined using the method previously described (102) with the following modifications: 100mm cell culture plates were seeded with 2×10^5 C₂C₁₂ cells transfected with either vector only or pcDNA3.1ID12 vector.

After 24 hours, plates were rinsed three times in 3 mL PBS and 5% LPDS media was added to the plate. Cells were incubated in LPDS media for 24 hours.

Cellular cholesterol determination

Cells were homogenized 20 strokes in CMF-PBS plus 0.2% triton, 100 μ M leupeptin and 100 μ M Pefa-bloc. Total cholesterol levels were determined by Dr. Steven Fliesler (St. Louis University School of Medicine, St. Louis, MO) using reverse-phase high-performance liquid chromatography (HPLC) after saponification and petroleum ether extraction, as described previously (18,19).

Immunofluorescence microscopy

Indirect immunofluorescence was performed on cells grown on collagen coated glass coverslips. 24 hours post-transfection, cells were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Cells were permeablized with 1% Triton X-100 in PBS for 5 minutes. Cells were then washed with PBST (Phosphate Buffered Saline and 0.1% Tween), and incubated with primary antibodies sheep anti-catalase (Calbiochem) and rabbit anti-IDI1 or IDI2 for one hour. After three washes, cells were incubated with fluorescently labeled secondary antibodies (Alexa-488 and Texas Red-X conjugated goat anti-sheep IgG antibody) for 1 hour at room temperature. Coverslips were mounted on glass slides in Mowiol 4-88 (Calbiochem) with 0.5% n-propylgallate as anti-fade agent. Fluorescence was visualized using confocal microscope (Leica).

Quantitative colocalization analysis

Confocal images were transferred to a Macintosh PowerPC G4 (Apple Computer, Cupertino, CA) for analysis. Colocalization of antigens was evaluated quantitatively for Pearson's correlation coefficient (R_r), Manders overlap coefficient using CoLocalizer Express (CoLocalizer Express Software, Boise, ID). Background was corrected using the threshold value for all channels to remove background and noise levels completely. Scatter grams were created that estimated the amount of each detected antigen based on colocalization of DsRed2/TexasRed (red, y-axis) and EGFP/Alexa 488 (green, x-axis). Colocalized pixels of yellow color were located along the diagonal of the scatter gram. At least three samples from each experiment were analyzed. Data were prepared as Excel and image files. Microsoft Excel software was used to analyze Excel files.

The text of Chapter Two, Experimental Procedures, in part, has been submitted for publication. The dissertation author was the primary researcher and author. The co-authors listed in this publication participated in the research which forms the basis for this chapter.

Characterization of IDI2, a Second Isopentenyl Diphosphate Isomerase in Mammals. Manuscript submitted. JBC. Clizbe D, Owens M, Shackelford J, Krisans SK.

Results

Identification and Isolation of Human IDI2 cDNA

In the course of performing a dBEST database search, we identified a 1.3 kB clone with an open reading frame of 684 nucleotides and 227 amino acids (figure 11) . The predicted protein product shows 81% similarity and 65% identity to human IDI, therefore, we designated this protein IDI2 (figure 12). A genome wide database search revealed the presence of ID12 in rat and mouse as well. A radiation hybrid panel assay located the human IDI1 and IDI2 genes in a tandem array on chromosome 10p15 (figure 13) in the interval pTEL-D10S558,26.92 cR3000 from the marker D10S1857. As reported earlier, the genes for IDI1(7 kb) and IDI2 (6 kb) lie 3.7 kb apart and have a similar exon-intron structure (figure 14). Both IDI1 and IDI2 consist of 5 exons, of similar size. In both genes the first exon is not translated.

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Human IDI1 ATGCCTGAAATAAACTAACCACCTCGACAAGCAACAGGTTCAACTCCTGGCAGAGATG
Human IDI2 ATGTCTGACATAAATCTTGACTGGGTGACAGGCGTCAGTTGCAGCGCTTGGAGGAAATG
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Human IDI1 TGTATCCTTATTGATGAAAATGACAATAAAATTGGAGCTGAGACCAAGAAGAATTGTCAC
Human IDI2 CTGATTGTTGTGGATGAGAAATGATAAGGTTATTGGTGCCGACACCAAGAGGAATTGCCAT
** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 CTGAACGAGAACATTGAGAAAGGATTATTGCATCGAGCTTTTAGTGTCTTCTTATTCAAC
Human IDI2 CTGAACGAAAACATTGAGAAAGGGCTGCTGCACCGAGCCTTCAGCGTTGTCTTGTTTAAC
***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 ACCGAAAATAAGCTTCTGCTACAGCAAAGATCAGATGCTAAGATTACCTTTCCAGGTTGT
Human IDI2 ACCAAGAATCGAATCTGATACAGCAGAGGTCGGACACGAAAGTCACGTTTCTGGGTAT
*** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 TTTACGAATACGTGTTGTAGTCATCCATTAAAGCAATCCAGCCGAGCTTGAGGAAAGTGAC
Human IDI2 TTTACCGACTCTGTAGTAGCCACCCATTATACAACCCAGCAGAACTGGAAGAAAAGGAT
***** * * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 GCCCTTGGAGTGAGGCGAGCAGCACAGAGACGGCTGAAAGCTGAGCTAGGAATTCCTTG
Human IDI2 GCCATCGGAGTGAGGAGGGCAGCCAGAGGCGCTGCAAGCAGAGCTGGGAATTCCTGGG
*** * ***** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 GAAGAGGTTCTCCAGAAGAAATTAATTATTTAACACGAATTCACACAAAGCTCAGTCT
Human IDI2 GAGCAGATTCTCCAGAGGACATTGTGTTTCATGACAATCTATCACCACAAGGCAAAATCA
** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 GATGGTATCTGGGGTGAACATGAAATTGATTACATTTTGTGTTGGTGAGGAAGAATGTAAT
Human IDI2 GACAGAAATTGGGGAGAGCATGAAATTTGTTACCTTCTGCTTGTGAGGAAAAACGTCAC
** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 TTGAATCCAGATCCCAATGAGATTAAGCTATTGTTATGTGTCAAAGGAAGAACTAAAA
Human IDI2 CTGAACCCGGATCCCAAGTGAACGAAAGCATCTCTACCTGTCCAGGAGGAGCTGTGG
***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 GAACTTCTGAAAAAGCAGCCAGTGGTGAAATTAAGATAACGCCATGGTTTAAATTTATT
Human IDI2 GAGCTGCTGGAGAGGGAGGCGAGGGGTGAAGTCAAAGTCACCCCTGGCTAAGAACCATT
** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 GCAGCGACTTTTCTCTTTAAATGGTGGGATAACTTAAATCATTTGAATCAGTTTGTGAC
Human IDI2 GCCGAGAGGTTTCTGTACCGTGGTGGCCTCACCTGGATGACGTGACCCCGTTTGTGGAG
** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Human IDI1 CATGAGAAAATATACAGAATGTGA
Human IDI2 CTTCACAAAATACAGAGTGTGA
* * * ** * ** * ** * ** *

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Figure 11. Nucleotide alignment of human IDI1 and IDI2. Identical nucleotides are indicated by (*).



Figure 12. Mammalian IDI1 and IDI2 show a significant degree of amino acid similarity. Amino acid sequence for hamster, rat, and human IDI1 and IDI2 are shown in Clustal W format. Catalytic amino acids are indicated by a bar (-); identical amino acids are indicated with an asterisk (*); conserved amino acids are indicated by (:) and semi-conserved by (.)

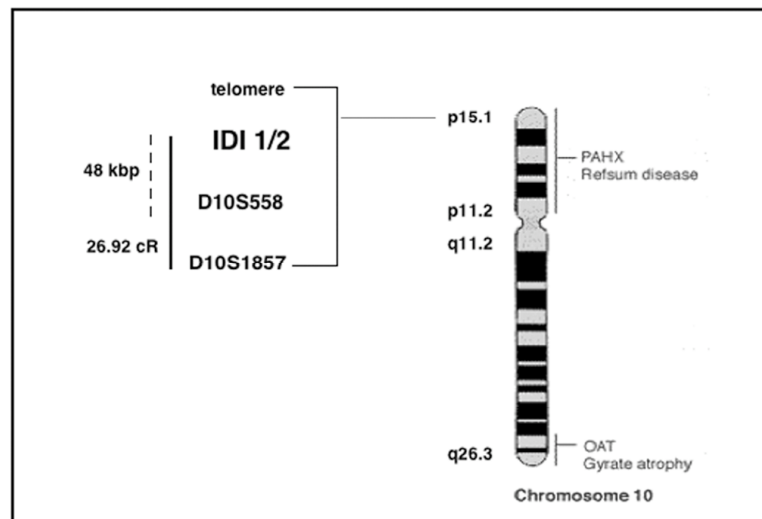


Figure 13. Human IDI1 and IDI2 are tandemly located on chromosome 10. Radiation hybrid panel assays indicate that IDI1 and IDI2 lie approximately 3.7 kb from one another on human chromosome 10p15. IDI1 is -26.92 cR3000 and IDI2 is located -5.23 cR3000 from IB3079 in the interval Chr10-pTEL-D10S558. Additionally, data provided from human genome project indicate the exact position of both genes is 48 kb telomeric units from the marker D19S558.

Promoter analysis

As a preliminary investigation into the possible regulation of the two genes we used the results of two recently published cDNA-microarray experiments which included IDI1 in their data sets (105,106). The first experiment monitored expression of a variety of genes in starved fibroblast cells following the addition of 10% serum both in the presence and absence of the translation inhibitor cyclohexamide. In this experiment IDI1 expression was arrested at 8 hours after addition of serum. Expression of IDI1 was strongly correlated with ribosomal protein L39 (RPL39), STE-20 related serine/threonine kinase (OSR1), and StAR-like protein (MLN 64). These results were confirmed in a second microarray experiment analyzing expression patterns of 60 different tumor cell lines under standardized culture conditions. These data confirmed the co-expression of IDI1, RPL39, OSR1, and MLN64. Thus, the results indicate that the co-expression is due to a common regulatory mechanism and is not merely an artifact of serum addition. To investigate the presence of a shared regulatory module in the promoter region of the four genes we employed the GEMS package (<http://genomatrix.gsf.de>). In this data set we identified two modules of transcription factor binding site with an interval of 95 to 250 bp. The first module contained a binding site for the cAMP response element binding protein (CREBP) family separated by 55 to 65 bp from a binding site for the sterol regulatory element binding protein (E-BOX/SREBP) family.

The second module contained a binding site for the E-BOX/SREBP family separated by 15 to 27 bp from a binding site for the SP1 family (figure 14).

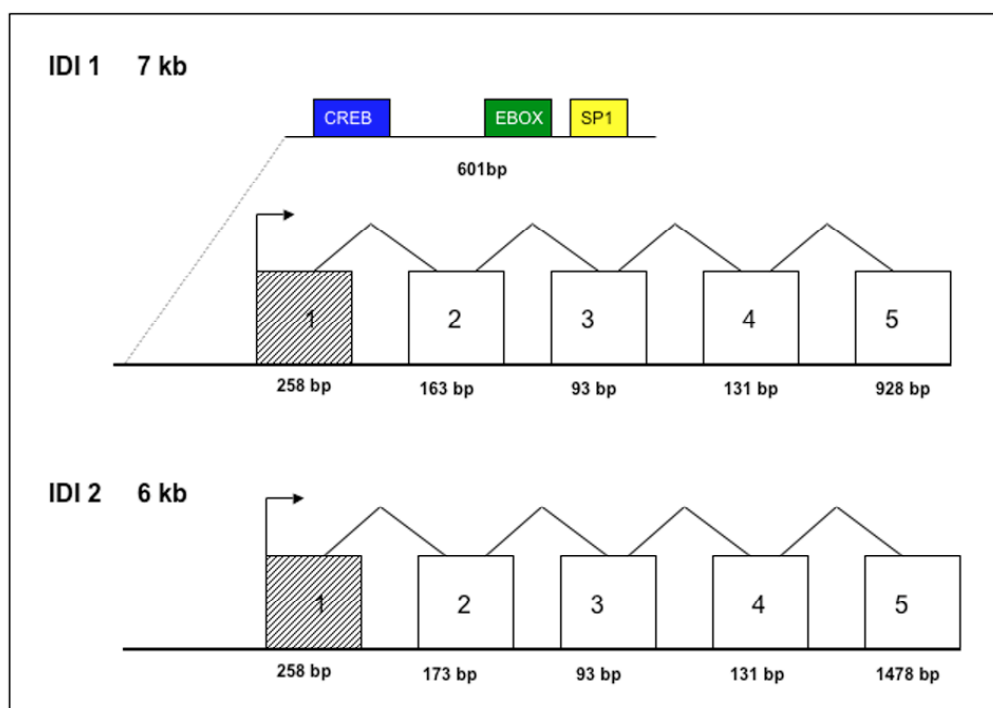


Figure 14. IDI genes have a similar structure. Gene structure of human IDI1 and IDI2 indicates that both each genes has five exons of similar size. The first exon of both genes is untranslated. Promoter analysis indicates that the 601 bp promoter region of IDI1 contains a common modular region with five other genes based on microarray experiments. The modules includes cAMP response element binding protein (CREBP), an EBOX/SREBP (EBOX) and a proximal binding site for SP1 family. These regulatory modules are absent in the promoter region of human IDI2.

This promoter model was present in all four promoters (IDI1, RPL39, OSR1, and MLN64), but not in any of the 1375 eukaryotic promoter sequences included in EPD Release 63 (825,000 bp in total)(107) Thus, our proposed promoter model is highly specific for the four co-regulated promoters. The absence of the proposed promoter model in the promoter region of IDI2 indicates that the two genes are most likely under independent regulation.

Tissue distribution of IDI2

In order to investigate the expression pattern of human IDI2 we probed a commercially prepared multiple tissue Northern blot (Clontech) against both IDI1 and IDI2 ³²P-labeled cDNA (figure 15). IDI1 expression is shown as a 2.4 kb transcript at abundant levels in skeletal muscle but is also detectable in brain, heart , kidney and liver (figure 15, panel a). In contrast, IDI2 expressed as a 1.9 kb transcript is only detectable in skeletal muscle (figure 15A, panel b). Simultaneous probing indicates that IDI2 is expressed at a higher level than IDI1 in skeletal muscle (figure 15A, panel c). The level of β -actin transcript was detected as an mRNA loading control (figure 15A, panel d).

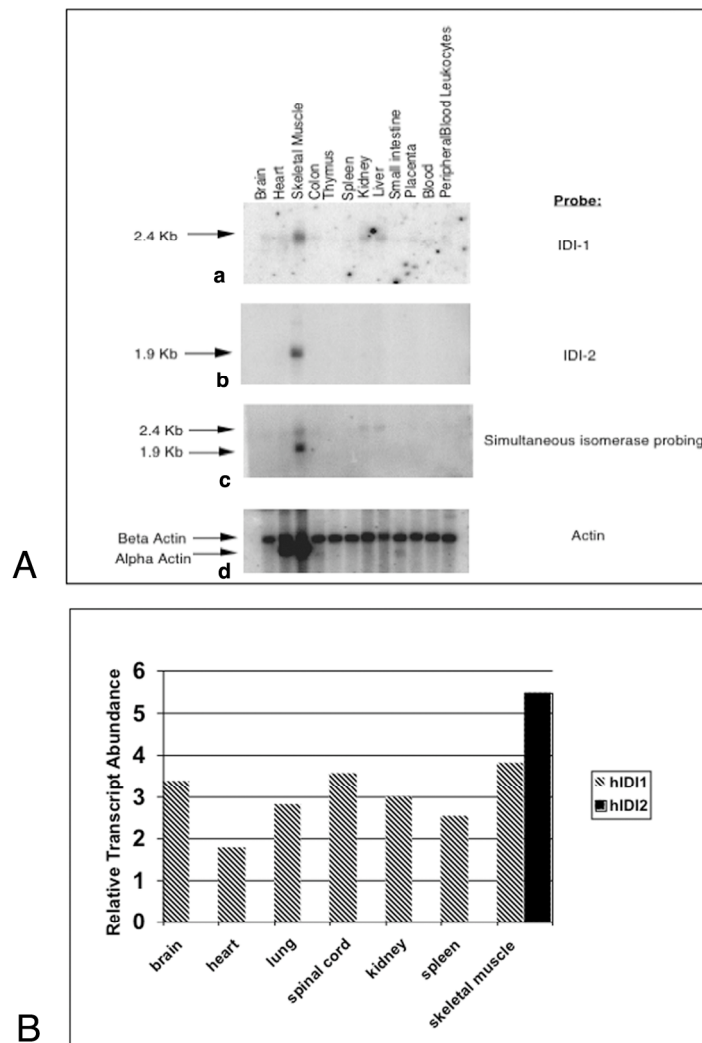


Figure 15. IDI1 and IDI2 show unique tissue expression patterns. (A) Multiple tissue Northern blot (Clontech) containing approximately 1 mg of poly(A) RNA per lane probed for IDI1 (panel a) and IDI2 (panel b). The same blot was hybridized with both IDI1 and IDI2 ^{32} P-labeled probe simultaneously illustrating higher expression for IDI2 in skeletal muscle (panel d). A ^{32} P-labeled actin cDNA probe was hybridized to the same blot to ensure equal loading of samples. Alpha actin, the predominate actin isoform, also hybridizes in sample lanes of heart and skeletal muscle. Adapted from K Masuda 2001.(B) Quantitative Real-time PCR (QRT-PCR) analysis of mRNA transcript expression across a variety of human tissues indicates that hIDI2 is expressed at detectable levels only in skeletal muscle. In contrast, hIDI1 is expressed across all tissues examined.

To further investigate the expression differences between the two genes we, in collaboration with Merck pharmaceuticals (Merck, Whitehouse Station, N.J., U.S.A.), analyzed expression of IDI1 and IDI2 using quantitative real-time PCR (QRT-PCR) in a variety of human tissues (figure 15 panel B). These data confirmed the previous findings and illustrate that while IDI1 is expressed ubiquitously in all tissues examined, IDI2 is expressed at detectable levels only in skeletal muscle. In agreement with Northern data, the QRT-PCR data illustrate that IDI2 is expressed at a 1.6x higher level than IDI1 in skeletal muscle.

Active site analysis in primates

It has been established that the isomerization of IPP to DMAPP occurs via an antarafacial protonation-deprotonation mechanism (108). Previous work has established the importance of two amino acids for the catalytic activity of IDI in yeast (65). The study identified glutamate E207, corresponding to E149 of the human enzyme, as a proton acceptor. This residue is conserved in both human isozymes. The second catalytically important residue identified as cysteine C139 of the yeast corresponding to C86 in the human enzyme is a serine S86 in IDI2. Mutational studies in yeast have shown that a C >A or a C >V change at this position abrogated catalytic activity. Importantly, a C > S mutation at this site resulted in an inefficient, yet still active, catalyst (65).

To investigate the presence of this S86 active site change in recent mammalian evolution we analyzed this specific active site region using PCR.

We amplified the 30 bp region encompassing the conserved S86 active site region of IDI2 in 3 species of primates using heterologous human IDI11 and IDI2 primers designed to flank the active site region (figure 16). Our data indicate that IDI2 is present in all species examined (figure 17).

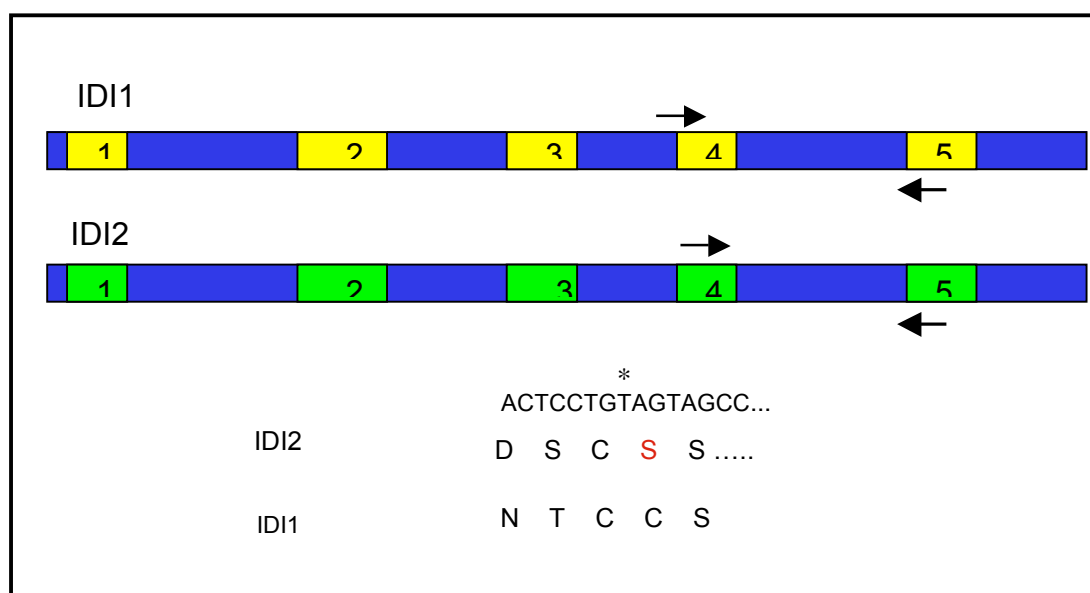


Figure 16. PCR amplification strategy for active site analysis in primate genome. Heterologous primers designed from human IDI1 and IDI2 flank exon 4 containing the putative active site. Amplicons generated from genomic DNA from primates, old and new world monkeys confirm evolutionary retention of active site differences.

Importantly, the C86S active site change is retained. Noteworthy is the degree of sequence similarity throughout the entire active site region for all primate species examined (100 %) as well as the high degree of sequence similarity for the entire IDI2 gene (98.97% human-chimpanzee, 98.13% human-gorilla). Thus IDI2 is highly conserved throughout primate evolution and is therefore likely to retain an important function.

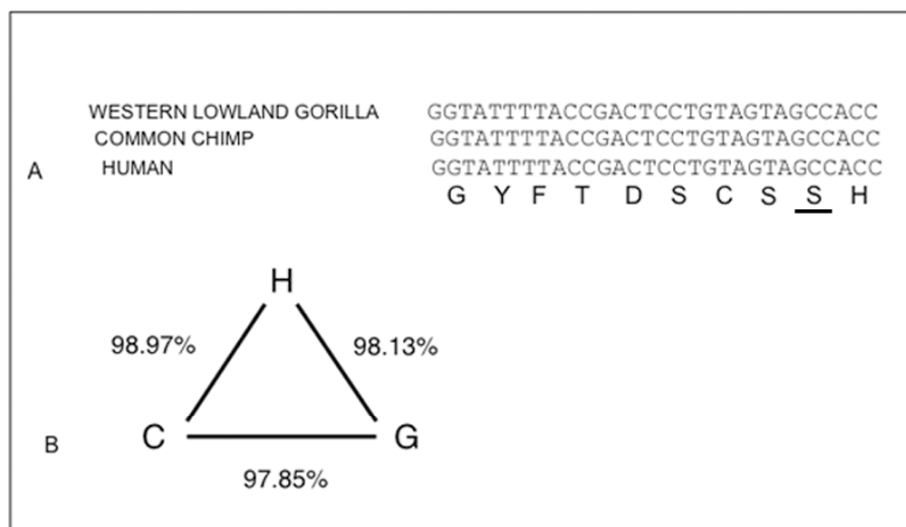


Figure 17. IDI2 C86S is conserved in primates. Active site analysis. The 30 bp IDI2 active site sequence analysis from chimpanzee and gorilla genomic DNA indicates that the IDI2 C86S mutation (underlined) is retained throughout evolution in mammals (panel A). Overall nucleotide identity within active site shows significant degree of conservation within primate species (panel B).

Recombinant IDI2 expression in *E. coli* and *S. cerevisiae*

Previously it had been shown in yeast IDI1 site-specific mutants that a C139S (C86S in human IDI2) mutation yielded an inefficient, yet viable, catalyst when tested for isomerase activity in vitro (65). Therefore, our aim was to investigate the ability of IDI2 to catalyze the isomerization of IPP to DMAPP. In addition, we were interested in the effect on the human IDI1 enzyme of a C86S change. To test this, we used site-directed mutagenesis to engineer constructs which contained a C86S in IDI1 and constructs that contained a S86C change in IDI2. We next designed an expression strategy based on the pET system (Novagen) that allowed subcloning into vectors containing N-terminal 6xHis tags enabling purification by Ni affinity column and detection of expressed

proteins using α -His antibodies. Initial attempts to isolate a soluble recombinant human IDI2 from pET 19 in *E coli* resulted in an insoluble protein which was incorporated into inclusion bodies (fig 18). As a result, in vitro measurements of activity were unreliable.

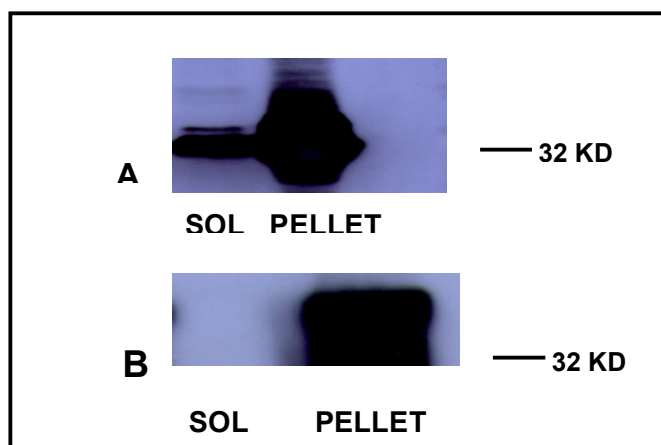


Figure 18. Recombinant IDI1 and IDI2 expressed in *E coli* result in an insoluble protein. (A) IDI 1 and IDI2 (B) expressed as a 32 kD fusion protein with an N-terminal 6XHis tag from a T7 promoter in pET 19 system (Clontech). Soluble fractions (SOL) resulted in very little (IDI1) or no soluble protein (IDI2). The majority of the expressed protein remained in inclusion bodies in insoluble pellets (PELLET). Proteins were detected with α -His antibodies (Roche).

In an attempt to express IDI1 and IDI2 as soluble proteins we used multiple strategies. Initially, we tested expression of the various pET 19 constructs in different *E coli* expression strains over various temperatures and growth times. We selected four *E. coli* expression strains including BL21(DE3), BL21(DE3), pLysS, BLR(DE3) and BLR(DE3)pLysS on the basis of their different phenotypes shown to increase expression and solubility. We performed expression of the cultures at three different temperatures: 37°C,

20°C and 15°C, as slowing down expression is well documented to improve protein solubility. Reduction in expression time or temperature had no effect on solubility (data not shown). Lastly, we used fusion proteins to improve the levels of expression and solubility. We tested the efficacy of three different fusion proteins on expression and solubility: NusA, Trx and GST. The final constructs include the following: fusion partner - 6XHis tag-target Protein. Fusion partners were either NusA, Trx or GST. Target proteins included (IDI1, IDIC86S, IDI2, IDI2 S86C). Expression of NusA fusion resulted in improved yet inadequate solubility for further reliable studies (figure 19). Trx and GST fusions remained completely insoluble (data not shown). We also attempted to improve solubility of the expressed proteins by removing the N-terminal 6xHis tag by protease cleavage from the pET 19 expressed protein, followed by guanadinium-HCl denaturation and renaturation. These results yielded a completely inactive protein for either IDI1 or IDI2 (data not shown).

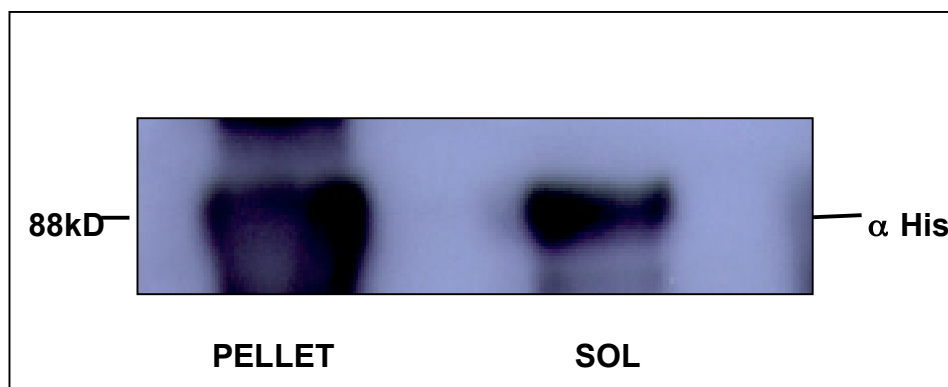


Figure 19. IDI2 expressed as a NusA fusion remains predominately insoluble. IDI2 expressed as a NusA fusion protein (88 kD) with an N-terminal 6xHis tag in *E. coli* remains bound as inclusion bodies. Proteins were detected with α -His anti bodies (Roche).

The possibility exists that hIDI2 may require posttranslational modification, since several myristoylation and glycosylation sites are predicted (Find Mod , Expasy). Thus we focused on a eukaryotic expression system in yeast for expression. We engineered hIDI1, IDI1C86S, hIDI2 and IDI2S86C as yeast expression constructs in pYES2.1 expression vector (Figure 20). We recovered soluble protein following expression of these constructs in diploid yeast INVSC1. Soluble His-tagged IDI proteins with a molecular weight of 31 kDaltons were detected by immunoblot analysis using anti-His antibodies (Figure 21 panel a).

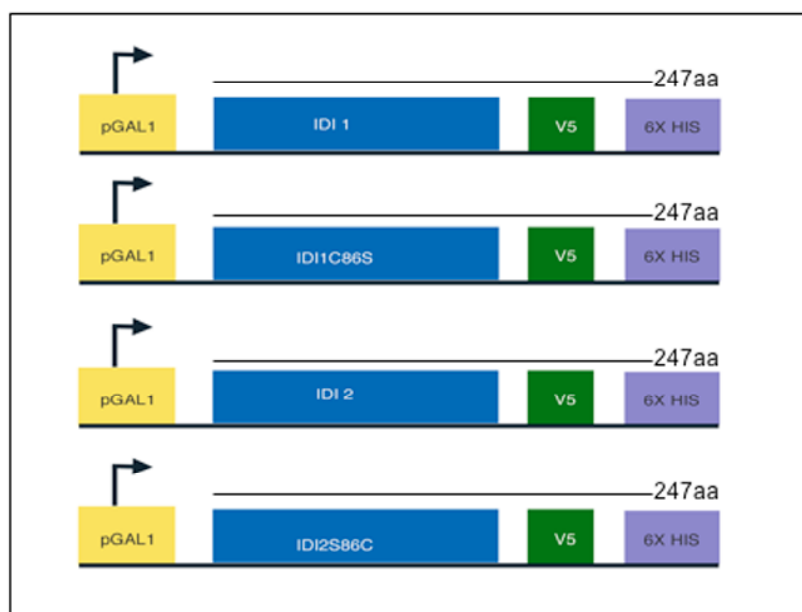


Fig 20. Yeast expression constructs Human IDI1, IDI C86S, IDI2 and IDI2S86C were cloned as cDNAs-fusion proteins with C-terminal V5 epitope and hexahistidine tag into pYES-V5-His TOPO. Expression of these fusion constructs are under the control of the Gal1 promoter that allows inducible protein expression in yeast in the presence of galactose.

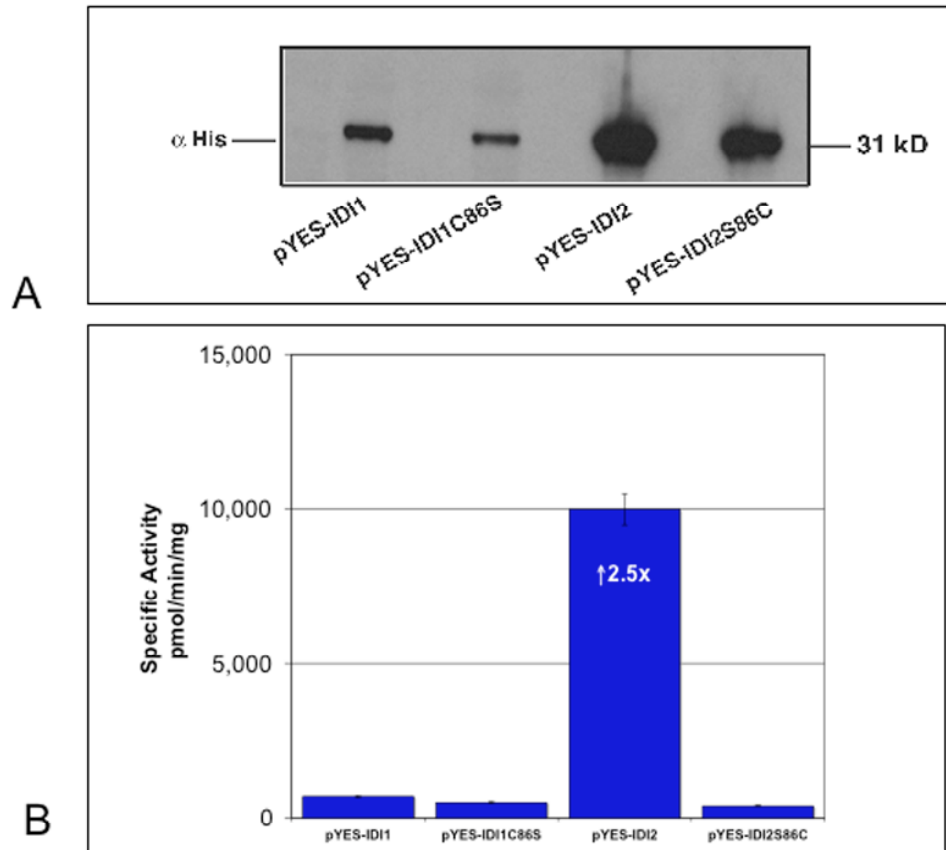


Figure 21. IDI2 has isomerase activity when overexpressed in *S. cerevisiae*. Panel A) Western blot of from pYES IDI recombinant constructs. Each lane contains 50 μ g total yeast cell extract expressing recombinant pYES IDI, pYESIDI1C86S, pYESIDI2, or pYES IDI2S86C. Proteins were detected with α -His antibody (Panel B) Recombinant IDI has isomerase activity when expressed in *S. cerevisiae*. Isomerase assay reactions containing 10 μ g protein from total cell extracts expressing recombinant IDI follows the conversion of acid labile conversion of ^{14} C-IPP TO ^{14} C-DMAPP. Background endogenous IDI1 values from overexpressed INVSC1 alone are subtracted. Results are the mean $\pm$ S.D. of three experiments.

Isomerase activity in IDI overexpressing INVSC1

To address whether the expressed proteins had isomerase activity we measured enzyme in vitro activity in total cell extracts compared to INVSC1 alone. The IDI1, IDI1C86S and IDI2S86C samples showed no significant activity above endogenous INVSC1 IDI activity alone (figure 21 panel b). IDI2 samples, however, showed a marked 2.5 fold increase above INVSC1 alone. As noted earlier the IDI1 and IDI1C86S cultures showed limited viability, therefore optimization of expressed protein concentration was difficult to control. However, in experiments in which IDI1, IDI1C86S and IDI2S86C protein volumes were increased 2-5 fold, only the IDI1 sample showed a concomitant increase in activity (data not shown). Thus, it is not reasonable to compare absolute activities between IDI1 and IDI2 as a measurement of enzyme efficiency. Rather we show that IDI2 has functional isomerase activity in vitro. In addition, neither IDI1C86S nor IDI2S86C sample showed any increase in activity with increased protein amounts. Therefore, the data demonstrate that the IDI1C86S and IDI2S86C mutants were inactivated by the amino acid change at position 86.

hIDI2 functional complementation of IDI1 in *S.Cerevisiae*

To address the ability of hIDI2 to functionally rescue a non-functional IDI1 in vivo in *S cerevisiae*, we employed the plasmid shuffle technique described by Hahn and Poulter, 1994. We transformed pYESIDI1 and pYESIDI2 into yeast strain FH2-5b by the method described. As shown previously, IDI1 is an

essential single copy gene in yeast, therefore the loss of pRS317:IDI1 is lethal unless functionally complemented by hIDI2. We were able to obtain double transformants for IDI2 (>30,000 colonies /transformation) by selection for uracil and lysine prototrophy. We were unsuccessful in recovering double transformants for human IDI1 despite repeated transformation attempts. Induced IDI2 transformants were then subjected to counter-selection by α -AA and further screened by replica plating on glucose or galactose media. True positive shuffle transformants were identified by PCR to confirm the presence or absence of pRS317:IDI1 or pYES IDI2 (figure 22, panel A).

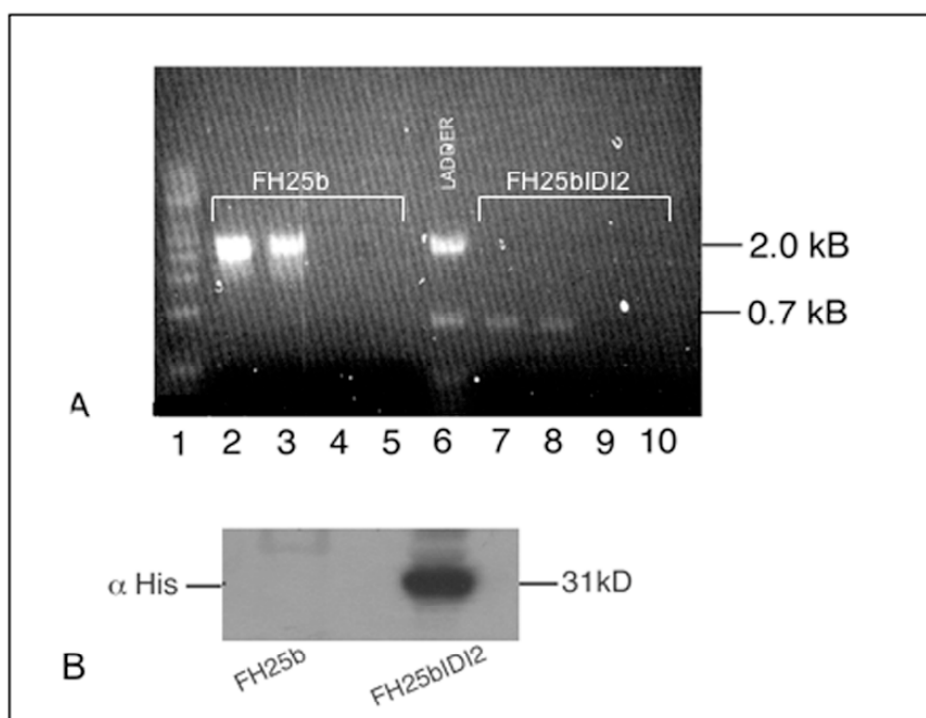


Figure 22. pYESIDI2 is efficiently shuffled into FH2-5b. Yeast colony PCR analysis (panel A) shows presence of IDI1 (lanes 2,3) and absence of IDI2 (lanes 4,5) in FH25b haploid yeast. In contrast, IDI1 is absent in FH25bIDI2 (lanes 9,10) and IDI2 is present (lanes 7,8). Lanes 1 and 6 contain DNA ladders for size reference. Western blot analysis of FH25b and FH25bIDI2 (Panel B) illustrate the presence or absence of 31 kD IDI2 in FH25b and FH25bIDI2 respectively when detected with α His antibody (Roche).

The presence of a 0.7 kb band in lanes 7 and 8 confirms the presence of pYES IDI2 in both colonies screened. The absence of a 2.1 kb band in lane 9 and 10 confirms the absence of pRS317:IDI1 in these colonies. As a control, non-transformed FH2-5b was screened for both pRS317:IDI1 and pYES IDI2 as well. Presence of a 2.1 kB band in lanes 2 and 3 confirms the presence of pRS317:IDI1 in FH2-5b. The lack of a 0.7 kb band in lanes 3 and 4 indicate the absence of pYESIDI2 in non-transformed F2-5b. We next tested whether we could detect IDI2 expression in transformed FH2-5bIDI2. Western analysis of the soluble protein indicates that 31 kD His-tagged IDI2 is expressed at high levels in FH2-5bIDI2 but not in FH2-5b (Figure 22 panel B). These results indicate human IDI2 can functionally complement yeast IDI1 in vivo.

Kinetic analysis of recombinant full length human IDI2 in FH2-5b

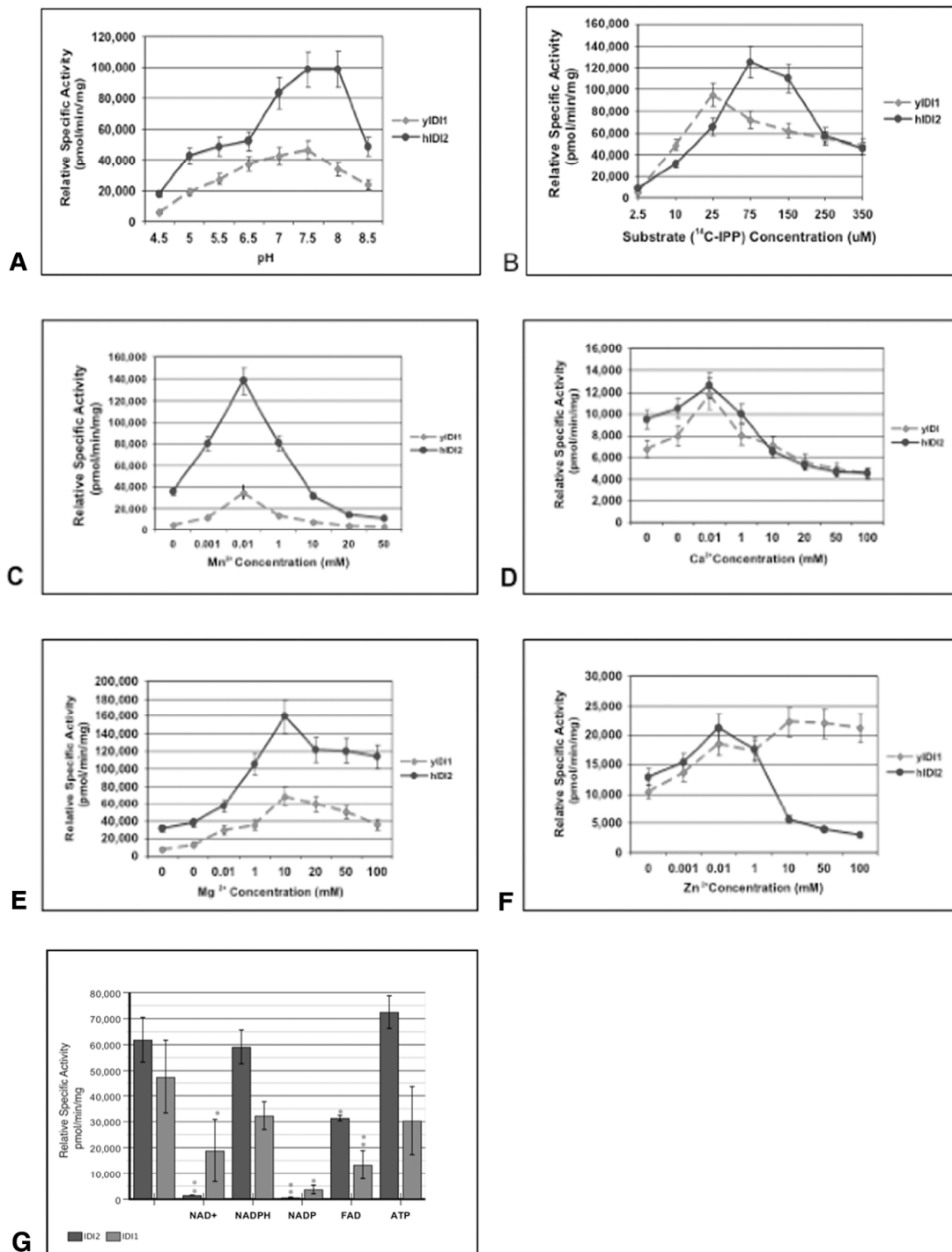
The recombinant yeast strain FH2-5bIDI2 offered the opportunity to investigate the catalytic activity of IDI2 in the absence of endogenous IDI1 and compare the kinetics with those previously characterized for IDI1.

To test the kinetic differences of over-expressed human IDI2 we grew cultures of FH2-5bIDI2 and recovered partially purified IDI1 and IDI2 by methods described. Presence of IDI2 was identified by Western blot and detected with anti-His antibody (data not shown). Partially purified IDI2 extracts were assayed for isomerase activity in vitro. Control assays of similarly prepared yIDI1 from cell lysates of FH2-5b were also measured. To determine optimal conditions for biological activity we first measured in vitro activity at various pH levels (figure 23 panel A). We show that hIDI2 has

maximal activity at pH 8.0. Thus all subsequent assays were carried out at pH 8.0 for hIDI2. We next determined the optimal substrate concentration for IDI2 at 75 μM ^{14}C -IPP compared to yIDI1 which shows optimal activity at a substrate concentration of 25 μM ^{14}C -IPP (figure 23 panel B). Thus, we determined hIDI2 maximal relative specific activity of $1.2 \times 10^{-1} \pm 0.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$ at pH 8.0 with a K_m^{IPP} value of 22.8 μM IPP.

Previous characterization of IDI1 in yeast showed that the enzyme requires a divalent metal as a cofactor for biological activity (85). Recently it has been shown that IDI1 isolated from *E. coli* has two metal binding sites, one site binds Mg^{2+} while the other is hypothesized to bind a Zn^{2+} atom (109). To determine the requirement for metal cofactors for hIDI2 we measured the *in vitro* activity of hIDI2 in the presence of several divalent metals (figure 23 panels C-F). The data suggest that hIDI2 is similar to yIDI1 in its requirement for Mg^{2+} , Mn^{2+} , Ca^{2+} . Notably however, the highest specific activity for IDI2 was seen at 0.01 mM Mn^{2+} compared to 10 mM Mg^{2+} , suggesting that Mn^{2+} may be the physiologically important cation. In addition, results show a marked difference in the two isozymes' requirement for Zn^{2+} (figure 23 panel F). Our data indicates that IDI2 has optimal activity at 0.01 mM Zn^{2+} , in contrast yIDI1 shows peak activity at 10.0 mM Zn^{2+} .

Figure 23. IDI2 has a different kinetic profile than IDI1. Isomerase activities of partially purified hIDI2 and yIDI1 were measured under conditions of increasing pH (4.5-8.5) (panel A) and substrate (2.5- 350 μM ^{14}C -IPP) (panel B). Partially purified hIDI2 and yIDI1 were tested for isomerase activity in vitro in the presence of different divalent metal cofactors including Ca^{2+} , Mg^{2+} , Mn^{2+} or Zn^{2+} (panels C-F). Various cofactors at 5 mM final concentration were added to the reaction mixture and tested for their effect on isomerase activity. The effect of flavin dinucleotides on IDI1 and IDI2 isomerase activity was tested (panel G). Isomerase activities were measured in vitro by detection of the conversion of acid labile conversion of ^{14}C -IPP TO ^{14}C -DMAPP. All reactions were performed in the presence of 75 μM ^{14}C -IPP at 37° C for 15 minutes using 10 μg partially purified yIDI1 and hIDI2 from FH2-5b or FH2-5bIDI2. Results are the mean $\pm$ S.D. of three experiments. t-test for significance indicates (*) $p \leq 0.05$, (**) $p \leq 0.01$.



As previously discussed, two distinct forms of IDI, type 1 and type 2 IDI, exist in nature. Despite catalyzing the interconversion of IPP to DMAPP Type 1 and type 2 IDI show no homology at the amino acid level. Type 1 IDI is utilized by nearly all eukaryotes and some bacteria, while type 2 IDI is present in all archaea as well as some bacteria including *Streptomyces* and *Bacillus*(86).

Recently it has been shown that type 2 IDI requires redox co-enzymes including FMN and NAD(P)H for activity (86). Although type 1 IDI has been shown to require only divalent metal ions we nonetheless were interested in measuring the requirement by IDI2, if any, for flavin cofactors. Thus, we measured the activity in vitro of partially purified hIDI2 and yIDI1 protein extracts from FH2-5b and FH2-5bIDI2 cultures in the presence of NAD^+ , NAD(P)H, FAD, NADH (figure 23, panel G). The data demonstrates that ,while neither isozyme showed an increase in activity in the presence of any of the dinucleotides tested, the effect of overall activity was dissimilar for IDI2 compared to IDI1. Specifically, IDI2 showed a significant decrease in relative specific activity in the presence of NAD^+ and NADP. In contrast, IDI1 relative specific activity showed a less significant effect in the presence of NAD^+ and NADP . However, in presence of FAD, IDI1 activity is affected to a greater extent than IDI2 .Taken together, our results suggest that the two isozymes are likely to have a different mechanism for isomerase activity.

hIDI1 and hIDI2 enzyme complexes

A great deal of evidence exists that many sequential metabolic enzymes, including those involved in the isoprenoid pathway in plants function as a multi-enzyme complex (110). To address whether IDI1 and IDI2 serve a similar concerted function we transformed diploid *S. cerevisiae* with pYESIDI1 and pYESIDI2 alone and together. Expressed proteins were detected with anti-His antibodies on a Western blot (figure 24). We then measured isomerase activity in cell extracts in vitro.

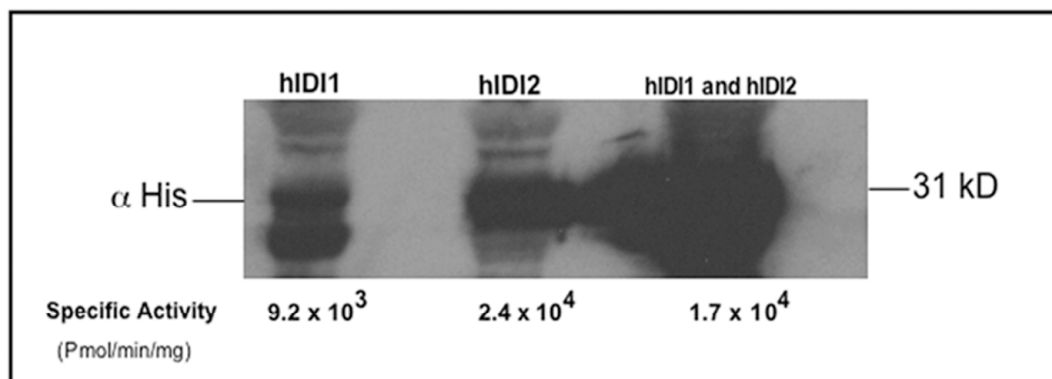


Figure 24. hIDI1 and IDI2 do not function in concert as an enzyme complex. Western blot of singly expressed hIDI1 and hIDI2 as well as co-expressed hIDI1 and hIDI2 in diploid INVSC1 cultures. Each lane contains 50 μ g protein from total cell extracts. 31 kD IDI proteins were detected with α -His antibody. In vitro isomerase activity was measured as conversion of 75 μ M ^{14} C-IPP to ^{14} C-DMAPP at 37°C for 15 min. Each assay reaction contained 10 μ g protein isolated from total *S. cerevisiae* strain INVSC1 cell extract of hIDI, hIDI2 and hIDI1-hIDI2 co-expressed cultures. Calculations for specific activity are based on total extracts including endogenous yIDI1 in INVSC1. Results are from representative experiment.

Our results indicate that co-expression of hIDI1 with hIDI2 had no detectable effect on overall isomerase activity (figure 24). Because we are

unable to discern the expression level of recombinant hIDI1 from expression levels of recombinant hIDI2 in co-expressed samples by Western analysis, we could not ensure the concentration of each protein in the dual expression samples. We, therefore, also tested isomerase activity in vitro of combined singly expressed extracts of hIDI1 and hIDI2 (data not shown). Results of combined individual extracts were similar to co-expression samples with no change in activity from singly expressed samples.

Subcellular localization of hIDI2

Recently it has been established, that IDI1 cloned from rat and hamster, is localized to the peroxisome by a pex5p dependent PTS1 signaling pathway (24). However as discussed earlier, many of the enzymes involved in the mevalonate pathway including HMG-CoA reductase, AA-thiolase have dual localizations (23, 20). Human IDI1 has a putative C-terminal PTS1 (-YRM) which conforms to the “two out of three” rule for the adherence to the canonical consensus sequence for peroxisomal targeting (111). In addition, the sequence of human IDI2 contains a less stringent C-terminal PTS1 (-HRV). Previously, it has been shown that many non-canonical tripeptide combinations were able to target the enzyme malate dehydrogenase in *S cerevisiae* to the peroxisome (111). Thus, we investigated the targeting and subcellular localization of hIDI1 and hIDI2 both as full-length proteins and as C-terminal tripeptide truncations in HeLa cells (figure 25).

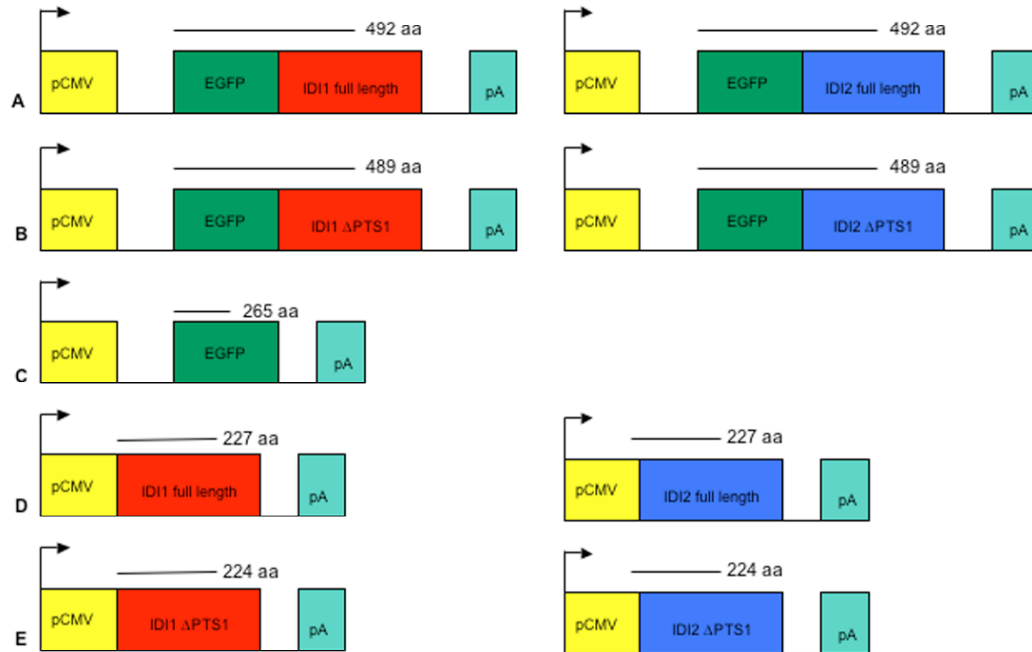


Figure 25. Targeting constructs. Full-length (A) and c-terminal tripeptide deleted (B) human IDI1 and IDI2 were cloned as C-terminal fusion proteins with EGFP followed by a poly-adenylation signal. EGFP expression construct is shown for size reference (C). Full-length cDNAs for human IDI1 and IDI2 were cloned into pcDNA3.1(-) under constitutive control of a CMV promoter and followed by a 3'-terminal poly-adenylation signal (D).

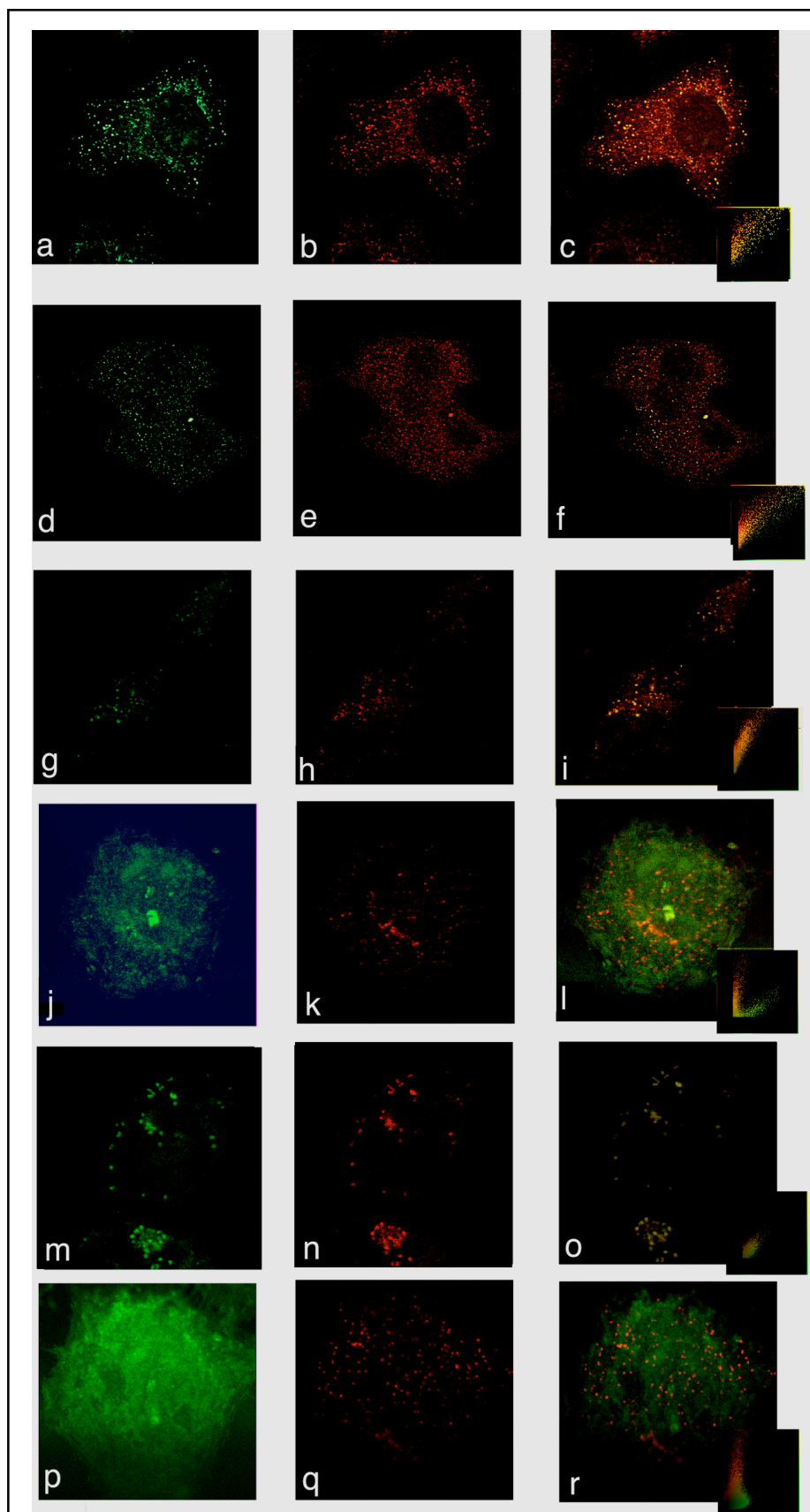
Stable transfections of full-length pCDNA3.1IDI1 and pCDNA3.1IDI2 into HeLa cells detected with antibodies against IDI1 or IDI2 show a punctate distribution indicative of a peroxisomal localization (figure 26 panels a and d). Furthermore, the patterns for IDI1 and IDI2 are superimposable with that of the peroxisomal marker catalase within the same cell (figure 26 panels c and f). To ensure that antibodies against IDI1 and IDI2 were specific for their cognate protein we also tested each antibody against non-transfected HeLa cells (data not shown). These results confirmed that the antibodies were specific and function only under conditions in which the protein is over-

expressed. Moreover, using software for quantitation of co-localization (Co-localizer Pro, Cupertino CA) we show that correlation values for colocalization (Pearson, R_r and Manders, R) indicate a significant degree of colocalization of both IDI1 ($R_r = 0.6608$, $R = 0.6488$) and IDI2 ($R_r = 0.6607$, $R = 0.6583$) with catalase. Scatter plots (embedded in lower right of figure 26 panels c and f) estimate the amount of each detected antigen based on colocalization of catalase (red, y-axis) and IDI1 or IDI2 (green, x-axis). Colocalized pixels of yellow color were located along the diagonal of the scatter gram. The scatter plots for full-length IDI1 and IDI2 indicate a monopartite diagonal scatter pattern, that again verifies the co-localization of both IDI1 and IDI2 with peroxisomal catalase.

To determine the requirement for the putative PTS-1 at the C-terminus of IDI1 and IDI2 for peroxisomal localization we generated EGFP fusion proteins of IDI1 and IDI2, both as full-length and C-terminal tripeptide PTS-1 deletion constructs (figure 26 panel A-C). In addition, we co-transfected these cells with the vector DsRed2peroxi (Clontech) which includes the coding sequence for the red fluorescent protein DsRed1 from *Discosomas p* followed by a C-terminal peroxisomal targeting sequence -SKL. Transient transfection of full-length EGFP-IDI1 and EGFP-IDI2 into HeLa cells resulted in a punctate labeling (figure 26, panels g and m) that was superimposable with the punctate pattern generated by pDsRed2peroxi (figure 26, panels h and n pDsRED2peroxi alone, panels i and o overlay of images). Correlation values for both EGFPIDI1/DsRed2peroxi ($R_r = 0.9098$, $R = 0.9099$)

EGFPID12/DsRed2peroxi ($R_r = 0.9454$, $R = 0.9454$) indicate a high degree of correlation. Scatter plots (figure 26 embedded lower right panels i and o) show a monopartite diagonal scatter pattern indicating overall correlation. In contrast, transfections of the truncated constructs pEGFPID11 Δ PTS1 and pEGFPID11 Δ PTS2 in HeLa cells resulted in a fluorescent pattern that was cytosolic in distribution (fig 26 panel j, panel p) while the fluorescent pattern for DsRed2peroxi remained punctate (figure 26, panels k and q). Moreover, the patterns of fluorescence were no longer superimposable for PTS-1 truncated constructs (figure 26, panels l and r). Colocalization correlation values for both EGFPID11 Δ PTS-1/DsRed2peroxi and EGFPID12 Δ PTS-1/DsRed2peroxi cotransfected cells were greatly diminished compared to full-length values ($R_r = 0.3099$, $R = 0.3098$; $R_r = 0.2671$, $R = 0.2670$). Scatter plots for these cells (figure 26, panels l and r) show a bipartite distribution along the x (green, EGFP) and y (red, DsRed2peroxi) axes indicating extremely low levels of overlap when truncated constructs are co-expressed with a peroxisomally targeted fluorescent protein.

Figure 26. IDI1 and IDI2 are localized to the peroxisome. Stably transfected HeLa cells were immunodetected with antibodies to IDI1 (panel a), IDI2 (panel d) or with antibody to peroxisomal marker enzyme catalase (panels b and e). A significant degree of the punctate pattern shown with hIDI1 and hIDI2 is superimposable over that obtained for the catalase antibody (panels c and f). Subcellular localization in HeLa cells of EGFP fusions of IDI1 and IDI2 full length (panels g, m) and PTS1 deleted (panels j, and p). Punctate fluorescent pattern is indicated by expression of DSRed2-peroxi in the same cells (panels h,k,n, and q). Peroxisomal colocalization of full-length IDI1 and IDI2 constructs is verified by superimposition of the two images (panels i and o). Overlay images indicate that deletion of C-terminal PTS1 for both IDI1 and IDI2 eliminates peroxisomal targeting (panels l and r). The embedded scattergrams generated by co-localization software (Co-localizer Express) indicates relative distribution of each fluorophore (lower right of overlay images c,f,i,l,o,r).



Regulation of IDI2 in mouse

As mentioned previously, the role of isoprenoids in skeletal muscle is not well characterized. However recent work has implicated a role for isoprenoids in skeletal muscle modifications following drug, exercise and dietary treatments. Our earlier work discussed the possibility that IDI2 may play a role in some muscle-specific pathologies including rhabdomyolysis in patients treated with statins, a class of cholesterol lowering drugs that acts specifically as HMG-CoA reductase (HMGCR) inhibitors (88). Another class of cholesterol lowering drugs, fibrates, modulates cholesterol levels through peroxisome proliferator-activated receptor (PPAR)- α . This PPAR- α activation is due to increased synthesis of lipoprotein lipase and apolipoprotein (apo) A-I. Interestingly, it has been shown that rhabdomyolysis is also a rare side effect of fibrate treatment. This effect has been shown to occur through a mechanism that involves a disruption in mitochondrial respiration (112). Additionally, isoprenoids have been implicated in modulating the autocrine function in skeletal muscle. The autocrine function of skeletal muscle has been shown to involve the expression and secretion of cytokines including IL-6, IL-8, IL-15. Activation of these cytokines is shown to be modulated through prenylated Ras. Interestingly these cytokines are induced following sustained and intense exercise (113). Moreover, changes in dietary regimes, including high fat and fasted diets, have been shown to modulate transcription of genes involved in isoprenoid and lipid metabolism in skeletal muscle (98,114).

Due to the muscle specific expression of IDI2 combined with the evidence of isomerase activity, we questioned whether IDI2 may play a unique role in the isoprenoid pathway in muscle. To address the effect of statin, fibrate, intense exercise treatments as well as high fat (HFD) and fasted diets, on transcription of IDI2 in skeletal muscle we designed a combinatorial experiment in which transcription levels of IDI1, IDI2 and HMG-CoA reductase (HMGCR) were measured by quantitative real time PCR (QRT-PCR) in murine muscle tissue (hind limb) following dietary or exercise treatment.

Our data indicate that IDI2 mRNA levels were significantly reduced (51% $p < 0.05$) under statin treatment (figure 27). In addition, IDI2 mRNA levels were significantly reduced (51%, $p < 0.05$) under fibrate treatment (figure 27). In contrast, mRNA levels of IDI1 and HMGCR were unchanged under either statin or fibrate treatment.

To address the effect of endurance exercise we measured murine IDI2 mRNA levels following a protocol to maximize endurance. Our analysis indicates that transcription under maximal exercise conditions was not affected for IDI1, IDI2 or HMGCR (figure 27).

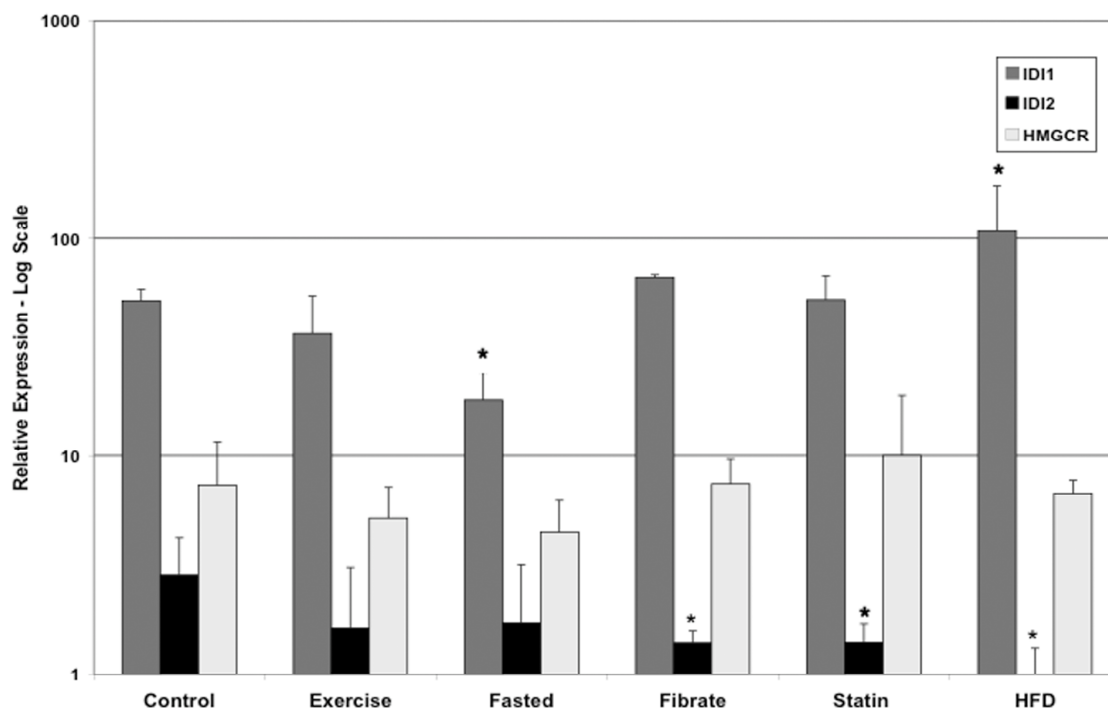


Figure 27. IDI1 and IDI2 are affected differently by diet, exercise and drug regimes. Quantitative Real-Time PCR analysis of mRNA expression levels of IDI, IDI2 and HMGCR measured in murine muscle following treatment with fibrate (Gemfibrozil), statin (Simvastatin) or following fasted, high fat diet (HFD) or exercise conditions. Relative expression is measured compared to untreated control mice (n=5). Results are the mean $\pm$ S.D. of three experiments. Mann-Whitney U test for significance indicates (*) $p \leq 0.05$. Adapted from Owens 2005.

We measured the effect of high fat diet (HFD) and fasting in skeletal muscle mRNA levels of IDI2 compared to IDI1 and HMGCR (figure 27). Our results indicate that IDI2 mRNA levels in skeletal muscle following HFD were significantly reduced (65%, $p \leq 0.05$), in contrast IDI1 transcription was increased significantly (209%, $p < 0.05$). Interestingly, measurements of mRNA transcription following a fasted regime indicate the significant downregulation of IDI1 (65%, $p \leq 0.05$). IDI2 and HMGCR transcription levels showed no significant effect. Taken together, these data suggest a regulatory difference for the three genes in skeletal muscle.

Murine IDI2 overexpression in C₂C₁₂ cells

To address the effect of murine IDI2 overexpression on sterol and non-sterol products downstream of the isoprenoid pathway, mammalian expression constructs designed to constitutively express either murine IDI1 or IDI2 were stably transfected into mouse myoblast C₂C₁₂ cells. C₂C₁₂ cells were chosen as a model system for overexpression studies because they are a well characterized mouse skeletal muscle myoblast cells which grow quickly, differentiate clearly and transfect easily. For over-expression studies, both murine IDI1 and IDI2 were cloned into an expression vector under control of a CMV promoter to generate high levels of cloned mRNA. Interestingly, we were unable to recover any viable IDI1 transformants. These results are similar to our attempts to overexpress hIDI1 in *S. cerevisiae* and are likely due to the intolerance for high levels of IDI1 in addition to endogenous IDI1 expression in these cells.

To address the effect of IDI2 over-expression in C₂C₁₂ cells we measured the rate of sterol (cholesterol) and non-sterol (dolichols, fatty acids) biosynthesis in murine IDI2 overexpressing C₂C₁₂ cells. Our results indicate that the rate of synthesis of cholesterol, fatty acids, and dolichols was decreased 38%, 31%, 38% respectively in IDI2 overexpressing cells compared to C₂C₁₂ cells stably transfected with pcDNA3.1(-) alone (figure 28, panel A).

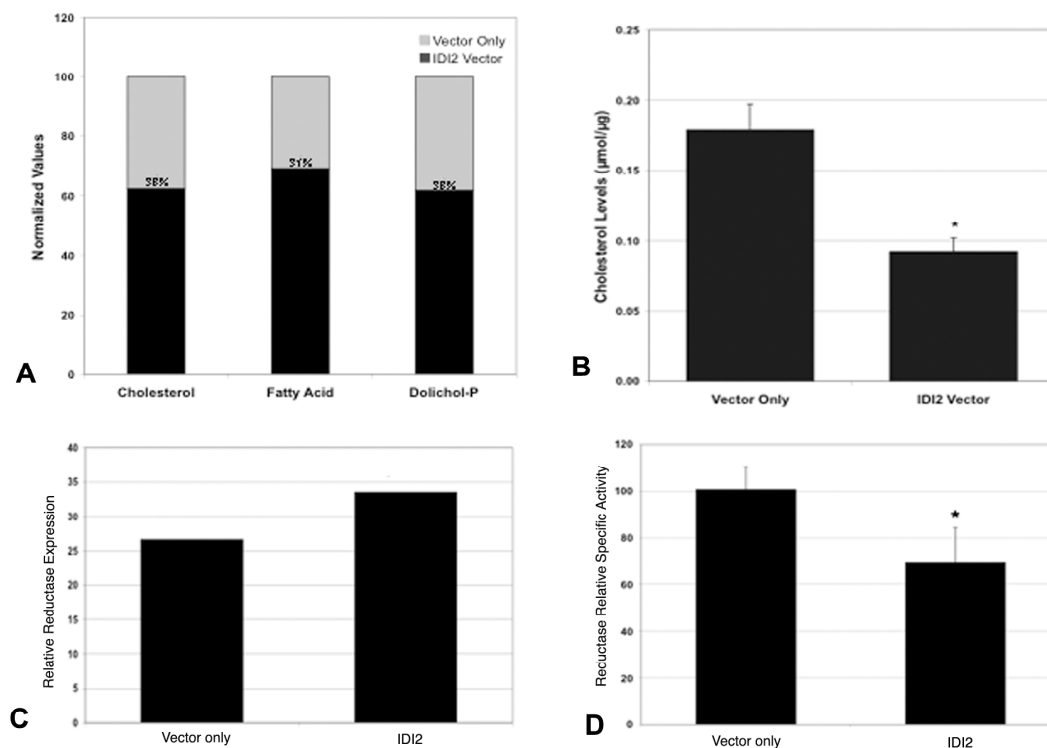


Figure 28. Cholesterol, fatty acid, dolichol-P synthesis and HMGCR are affected differently by the overexpression of IDI2 in mouse myocytes. Cholesterol, fatty acid, and dolichol-P synthesis was measured in IDI2 stably transfected C₂C₁₂ cells by incorporation from ¹⁴C-labeled acetate (panel A). Total cellular cholesterol levels were determined by reverse-phase HPLC (panel B) Normalized values are measured compared to stably transfected vector only control C₂C₁₂ cells. Results are the mean $\pm$ S.D. of three experiments. t-test for significance indicates (*) $p \leq 0.05$. Real-time PCR measured HMGCR mRNA levels in murine IDI2 and vector stably transfected C₂C₁₂ cells (panel C). Experiments were performed in triplicate. Results are from a representative experiment. HMGCR activity measured in stably transfected vector only and IDI2 transfected C₂C₁₂ cells (panel D). Experiments were performed in triplicate. Relative activity is measured compared to vector control cells. Results are the mean $\pm$ S.D. of three experiments. t-test for significance indicates (*) $p \leq 0.05$. Adapted from M Owens 2005

Additionally, we measured the amount of total cellular cholesterol in murine IDI2 overexpressing cells compared with those cell stably transfected with pcDNA3.1(-) alone (figure 28, panel B). The data demonstrate that, similar to the synthesis data, total cholesterol is decreased in IDI2 over-expressing C₂C₁₂ cells 38% compared to vector transected control cells.

To address the possible mechanism for reduction of cholesterol by IDI2 over-expression, we measured the level of HMGCR transcription (figure 28, panel C) as well as HMGCR activity in C₂C₁₂ cells (figure 28, panel D). Interestingly, HMGCR mRNA levels were slightly increased compared to pcDNA 3.1(-) vector transfected control cells, however HMGCR activity measured in both cells show a significant reduction in HMGCR activity (31%, $p<0.05$). These data suggest that IDI2 may play a role in the modulation of downstream sterol and non-sterol products and this regulation is likely at the level of HMGCR inhibition.

The text of Chapter Three, Results, in part, has been submitted for publication. The dissertation author was the primary researcher and author. The co-authors listed in this publication participated in the research which forms the basis for this chapter.

Characterization of IDI2, a Second Isopentenyl Diphosphate Isomerase in Mammals. Manuscript submitted. JBC. Clizbe D, Owens M, Shackelford J, Krisans SK.

Discussion

Our work describes a novel mammalian isozyme, IDI2. IDI2 shares a high degree of sequence homology to its isomer IDI1 at both the amino acid and nucleotide level. Structural analysis indicates that both isozymes have open reading frames of similar size with nearly identical intron and exon boundaries. In addition, the first intron of both genes is not translated. Further analysis of chromosomal localization indicates that both genes lie in tandem on the petite arm of chromosome 10. We have previously described the likelihood that IDI2 arose as an ancient tandem duplication event approximately 70 mya, prior to the evolutionary divergence of mouse and humans. Several instances of multiple isoforms of IDI1 are seen in plants where differences in subcellular compartmentalization confer a mechanism for regulation. Promoter analysis on the two isozymes indicate that the IDI1 promoter region contains a binding site the EBOX/SREBP members of transcription factors. Interestingly, this promoter module is absent in the promoter region of IDI2 indicating that the two genes are likely regulated by independent mechanisms.

We recently described the differences in expression pattern for the two isozymes in mouse and human based upon EST sequence comparisons (88). Here we describe an in-depth analysis of IDI1 and IDI2 in humans at the level of transcription. By both Northern analysis and QRT-PCR in a variety of human tissues, we show that at the mRNA level IDI1 is ubiquitously expressed in all tissues examined. In contrast, IDI2 is expressed at detectable levels only

in skeletal muscle. Thus, we expect that IDI2 may play a muscle specific role in isoprenoid metabolism.

Previously, the active site amino acids for IDI1 were identified by comprehensive mutational analysis in yeast (65). Those analyses identified Glu207 (Glu149 in human) and Cys139 (Cys86 in human) as the catalytically active residues. Interestingly, that study reported that while a Cys>Ala and Cys>Val completely ablated activity, a Cys>Ser change of that position resulted in an inefficient, yet still active, enzyme. Activity analysis presented here indicates that, in a yeast expression system, hIDI2, which retains the Glu149 critical residue but has Cys86>Ser86 mutation, has isomerase activity in vitro. Moreover, hIDI2 can functionally complement *idi1* yeast in vivo. Interestingly, when tested in vitro for isomerase activity, a Ser86>Cys86 mutation in IDI2 resulted in a loss of activity when measured in vitro.

Our earlier study proposed a possible model for isomerase activity in IDI2 (88). In this model, we proposed that the exchange of Asn83 by Asp83 may sufficiently increase the acidity of serine 86 to enable protonation of IPP. Furthermore in a recent study characterizing the crystal structure of the inactive C67A in *E. coli* complexed with the irreversible inhibitor EIIP, Wouters et al show that the protonation machinery is still intact and that under certain conditions Glu116 (Glu 149 human) can function as an active nucleophile (115). Thus, we expect that IDI2 is capable of catalyzing the isomerization of IPP to DMAPP by a novel mechanism.

Our study shows that IDI2 has a maximal relative specific activity of $1.2 \times 10^{-1} \pm 0.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$ at a pH optima of 8.0 at 37°C. This value is 34-fold lower than published values for human IDI1 ($V_{\text{max}} 4.1 \pm 0.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ at pH optima of 7.0) (61). This difference in activity for the two enzymes reflects an inherently different catalytic mechanism.

The two isozymes demonstrate a distinct difference from one another in their requirement for Zn^{2+} . Recently, it has been shown that Zn^{2+} is an essential cofactor for IDI1 (109). Presumably, Zn^{2+} provides a structural and possibly a catalytic role for the enzyme thus the differences in the Zn^{2+} requirement of the two isozymes likely reflects differences in catalytic mechanisms.

Additional catalytic differences are noted in the reduction of activity in the presence of various redox cofactors: NAD^+ , NADP^+ , FAD and NADPH. The role for redox cofactors is diverse; these cofactors perform complex redox chemistry, provide an important link in signal transduction, and participate in crucial electron transfer pathways. It is not surprising, given the generally accepted protonation-deprotonation mechanism for isomerization, that activity was significantly reduced for both isozymes. In fact, the most probable explanation is that the positively charged oxidized cofactors interact with the negatively charged glutamate within the active site resulting in a diminution of catalytic activity. Interestingly, IDI1 is affected significantly more by FAD while IDI2 is affected significantly more by the presence of charged NAD^+ and NADP^+ . It is well known that NAD^+ and NADP^+ function as classical coenzymes while the

mode of action of FAD requires the molecule to bind tightly as a prosthetic group. The effective differences of the various cofactors, therefore, reflects a dissimilarity in affinities for these redox species and implies a distinctive catalytic mechanism for IDI2 compared to IDI1.

As described earlier, it has been shown that IDI is also found in some organisms that synthesize both IPP and DMAPP by an alternative pathway from methyl-erythritol phosphate (MEP) (114). It is hypothesized that IDI in these organisms may function in maintaining a balance of IPP and DMAPP pools for subsequent synthesis reactions (114). We examined the possibility that IDI2 may serve a similar role by concerted action with IDI1. We found, however, that the two isozymes are not likely to function as a multimer, as *in vitro* combined extracts demonstrated no concomitant change in activity. Thus, we expect that IDI2 does not function as a regulator of IDI1 activity in skeletal muscle.

Further characterization of the two human isozymes indicates that both IDI1 and IDI2 are localized to the peroxisome by way of a pex5p-dependent import pathway. In our current study we show that C-terminal tripeptide deletions of a putative PTS1 sequence (-YRM, IDI1 and -HRV, IDI2) results in the loss of peroxisomal targeting of EGFP-fusion proteins in HeLa cells. These results are not unexpected in light of the recent evidence expanding the canonical C-terminal PTS1 tripeptide to include the residues {SAGCN}-{RKH}-{LIVMAF}(117). Importantly, it has been shown that even non-conserved substitutions of the -3 residue position still retain peroxisomal targeting function

(111). Recent evidence for this was shown in the characterization of rat and hamster IDI1 (72). In this study it was shown that the C-terminal tripeptide – HRM is necessary and sufficient for peroxisomal localization. Recent studies have determined that cryptic upstream auxiliary residues at positions -4 and -5 affect pex5p receptor binding affinities and thus peroxisomal import (118). Importantly, sequence analysis indicates that human IDI1 and IDI2 share complete amino acid homology at these positions with peroxisomal rat and hamster IDI1 (I, -4 and K, -5). While differences in subcellular localization of IDI1 in plants are thought to provide a mechanism for regulation of the various isoforms, our data suggest no such organizational mechanism exists for the IDI isoforms in human. Regulatory differences for the two isozymes may therefore involve differences in promoter region and transcription factor interactions.

Presently, the metabolic effect of dietary and cholesterol lowering regimes on the transcription of skeletal muscle genes in the mevalonate pathway is unknown. However many of the effects of these treatments in liver have been well described (119,120). Our current study demonstrates that two, well characterized, genes of the mevalonate pathway, HMGCR and IDI1, as well as a novel muscle specific isoform IDI2, show a different transcriptional sensitivity to dietary, exercise and cholesterol lowering treatments in murine muscle than has been described in liver. Notably, IDI2 is significantly affected by treatment with cholesterol lowering treatments statins and fibrates. It has been shown that fibrates modulate synthesis of lipoprotein lipase and ApoA1 mediated by PPAR α , a class of nuclear transcription factors involved in regulation of lipid

metabolism. Similarly, statins have been shown to upregulate liver fatty acid-binding protein (L-FABP) through PPAR α dependent activation (121). Dietary modifications including HFD and fasting have also been implicated as activators of PPAR α (122). Therefore the possibility exists that IDI2 transcription is modulated by PPAR α activation.

For over four decades HMGCR has been shown to be the rate-limiting enzyme in the mevalonate pathway and thus serves to regulate the biosynthesis of isoprenoids. Competitive inhibitors of HMGCR block the conversion of HMG-CoA to mevalonate and thereby inhibit cholesterol synthesis in the liver. Regulation of HMGCR is complex and multifaceted and is known to occur at the transcriptional, translational, and post-translational level. Sterols have been shown to regulate HMGCR post-transcriptionally by increasing the rate of degradation (123). Moreover recent evidence suggests that non-sterol species including the C₁₅ isoprenoid FPP or farnesol (FOH) may be regulators of HMGCR (3). Thus, the likelihood exists that the mechanism for the decrease in HMGCR activity and resultant decrease in downstream products, cholesterol and dolichol, in the IDI2 over-expressing C₂C₁₂ cells is through an increased pool of FPP and /or FOH. Moreover, FOH has been shown to induce activation of PPAR α (3). Thus the possibility exists that regulation of IDI2 may be through a feedback mechanism involving FPP and/or FOH production and PPAR α activation in skeletal muscle.

Fatty acid and cholesterol biosynthesis are strongly associated. Both are coordinately regulated by sterol element-binding proteins (SREBPs) and liver X receptors (LXRs). LXRs form obligate heterodimers with another nuclear hormone receptor retinoid X receptor (RXRs). As heterodimers these transcription factors bind to response elements in target genes, including several cholesterol biosynthesis genes (124). Previous studies have shown that the addition of the C₂₀ isoprenoid, geranylgeraniol pyrophosphate (GGPP) as well as its alcohol derivative geranylgeraniol, inhibit LXR activation. As a result, transcription of target genes involved in cholesterol efflux are inhibited (13). More importantly, recent studies have indicated that FPP, which is interconvertible with FOH, inhibits fatty acid synthesis in an SREBP independent manner (125). Thus, the results of our current study indicating that over-expression of IDI2 in murine skeletal muscle cells results in a inhibition of HMGCR activity, total cholesterol and rate of cholesterol synthesis are likely due to a resultant increased pool of available FPP.

In conclusion, we have identified a novel isozyme of isopentenyl diphosphate isomerase, IDI2, in humans. Our data demonstrate that IDI2 differs from previously characterized IDI1 in its tissue distribution, kinetic parameters, and catalytic mechanism. We show that although both isozymes are localized to the same subcellular compartment they are regulated independently from one another. Moreover, we demonstrate that, unlike IDI1, which is regulated at the level of SREBP, IDI2 is likely to be regulated by a different mechanism, one that may involve activation of the nuclear transcription factor PPAR α . The muscle

specific transcription of IDI2 along with the enzymes role in modulating cholesterol biosynthesis through HMGCR inhibition makes IDI2 an interesting candidate as a cholesterol lowering therapeutic target.

The text of Chapter Four, Discussion, in part, has been submitted for publication. The dissertation author was the primary researcher and author. The co-authors listed in this publication participated in the research which forms the basis for this chapter.

Characterization of IDI2, a Second Isopentenyl Diphosphate Isomerase in Mammals. Manuscript submitted. JBC. Clizbe D, Owens M, Shackelford J, Krisans SK.

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