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Dual compartmental localization and function of mammalian NADP⁺-specific isocitrate dehydrogenase in yeast

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Abstract

Isozymes of NADP⁺-specific isocitrate dehydrogenase (IDP) provide NADPH in cytosolic, mitochondrial, and peroxisomal compartments of eukaryotic cells. Analyses of purified IDP isoforms from yeast and from mouse suggest a general correspondence of pH optima for catalysis and *pI* values with pH values reported for resident cellular compartments. However, mouse IDP2, which partitions between cytosolic and peroxisomal compartments in mammalian cells, exhibits a broad pH optimum and an intermediate *pI* value. Mouse IDP2 was found to similarly colocalize in both cellular compartments when expressed in yeast at levels equivalent to those of endogenous yeast isoforms. The mouse enzyme can compensate for loss of yeast cytosolic IDP2 and of peroxisomal IDP3. Removal of the peroxisomal targeting signal of the mouse enzyme precludes both localization in peroxisomes and compensation for loss of yeast IDP3.

Keywords

Isocitrate dehydrogenase; *Saccharomyces cerevisiae*; compartmentalization; NADPH; β -oxidation

Introduction

Eukaryotic cells have several genetically distinct isocitrate dehydrogenase enzymes that catalyze the oxidation of isocitrate to α -ketoglutarate with concomitant production of NADH or NADPH. The NAD-specific mitochondrial enzyme is structurally complex, allosterically regulated, and functions in the tricarboxylic acid cycle. In contrast, differentially compartmentalized NADP-specific enzymes (IDPs¹) are simple homodimers that are not allosterically regulated. Mitochondrial enzymes (designated IDP1s) are ancillary sources of α -ketoglutarate and mitochondrial NADPH [1,2], and yeast IDP1 is constitutively expressed [3]. Non-mitochondrial IDPs apparently function to provide NADPH for biosynthetic reactions and for thiol-based antioxidant systems.

In *Saccharomyces cerevisiae*, there are two non-mitochondrial IDP isoforms that are 68% identical [4-6]. IDP2, a cytosolic enzyme, is expressed with carbon sources other than glucose and after the diauxic shift when glucose is exhausted in the medium [7,8]. Although disruption

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¹*Abbreviations used:* IDP, NADP-specific isocitrate dehydrogenase; IDP1, mitochondrial isocitrate dehydrogenase; IDP2, cytosolic isocitrate dehydrogenase; IDP3, peroxisomal isocitrate dehydrogenase; mamIDP2, mammalian cytosolic/peroxisomal isocitrate dehydrogenase

of the *IDP2* gene alone produces no dramatic growth defects, co-disruption of *IDP2* and *ZWF1*, the yeast gene encoding glucose-6-phosphate dehydrogenase which is the rate-limiting enzyme in the hexose monophosphate pathway, produces not only an inability to grow but, in fact, a rapid loss in viability upon shifting an *idp2Δzwf1Δ* strain to medium with a fatty acid carbon source [9]. β -oxidation is strictly a peroxisomal process in yeast [10] and, for each round of the pathway, there is a stoichiometric production of hydrogen peroxide in first reaction catalyzed by acyl-CoA oxidase. While the presence of either cytosolic enzyme, *IDP2* or *ZWF1*, is permissive for growth in medium with a fatty acid carbon source, a detrimental accumulation of hydrogen peroxide occurs in an *idp2Δzwf1Δ* strain presumably due to inadequate production of NADPH for cytosolic antioxidant systems that normally aid in removal of that byproduct of peroxisomal β -oxidation [11].

The other non-mitochondrial NADP-specific yeast enzyme is *IDP3*, a peroxisomal enzyme targeted via a type I peroxisomal targeting sequence (a Cys-Lys-Leu tripeptide at the carboxyl terminus) [5,6]. *IDP3* expression is induced by growth with fatty acid carbon sources, and *IDP3* is required to produce the NADPH required for a reductive step in β -oxidation catalyzed by 2,4-dienoyl-CoA reductase [5,6]. This reaction is specifically needed to reposition double bonds that follow even-numbered carbon atoms in some fatty acids, e.g. petroselinic and docosahexaenoate², prior to further processing by β -oxidation. Thus, a yeast mutant lacking *IDP3* is unable to use such fatty acids as carbon sources, whereas growth with fatty acids lacking such double bonds, e.g. oleate, is unaffected.

In contrast to yeast, mammalian cells contain a single non-mitochondrial isozyme of *IDP* (*mamIDP2*) that localizes in both the cytosol and in peroxisomes [12,13]. The mammalian enzyme shares sequence identities of 64% with yeast *IDP2* and 57% with yeast *IDP3*, and it presumably fulfills the biochemical functions of both of its yeast counterparts. In cell culture systems, overexpression or reduced expression of *mamIDP2* has been shown to result in, respectively, protection or susceptibility to damage from oxidant or radiation challenge [14, 15]. There is also evidence for functions of *mamIDP2* in provision of NADPH for lipid biosynthesis in the cytosol [16] and in metabolite/cofactor shuttle cycles between the cytosol and mitochondria [17] and between the cytosol and peroxisomes [18]. *MamIDP2* contains a type I peroxisomal targeting sequence (an Ala-Lys-Leu tripeptide at the carboxyl terminus) [13,19]. However, mechanisms and conditions influencing the co-distribution of the enzyme in two different cellular compartments are unknown.

In the current report, we compare kinetic properties and other attributes of *mamIDP2* with those of yeast *IDP2* and *IDP3* isozymes (and with corresponding mitochondrial *IDP1* isozymes) to identify characteristics that might correlate with functions in different cellular compartments. Also, to begin to analyze factors affecting compartmental distribution of *mamIDP2*, we expressed both the authentic enzyme and a form of the enzyme (*mamIDP2*^{ΔAKL}) lacking the peroxisomal targeting signal in yeast at levels comparable to those of the yeast isozymes. We examined the distribution of *mamIDP2* in the cytosol and in peroxisomes in yeast cells and determined the extent that physiologically relevant levels of *mamIDP2* can compensate for loss of either or both yeast isozymes.

Materials and methods

Expression, purification, and kinetic analyses of *IDP* isozymes

Mouse liver *IDP1* and *IDP2* cDNAs (I.M.A.G.E. clones, American Type Culture Collection) were amplified using polymerase chain reaction (PCR) with primers designed to insert codons

²Fatty acids used in current and previous studies are: (9) oleic acid (C18:1), (6) petroselinic acid (C18:1), and (4,7,10,13,16,19) docosahexaenoic acid (C22:6).

for six histidine residues onto the 3' ends of the coding regions and subcloned into pET15b plasmids (Novagen) for expression in *Escherichia coli* strain BL21(DE3). All constructs were verified by DNA sequence analysis. Bacterial transformants were grown in Luria Broth at 37°C to an $OD_{600nm} = 0.5-1.0$. Protein expression was induced by adding isopropyl β -D-thiogalactopyranoside to 1.0 mM, and incubation was continued for 12 h at 25°C for the mamIDP1 protein or for 6 h at 30°C for the mamIDP2 protein. Yeast *IDP1*, *IDP2* and *IDP3* genes also containing codons for 3' histidine tags were subcloned into multicopy two-micron plasmids for expression in a *S. cerevisiae* mutant strain (*idp1 Δ idp2 Δ idp3 Δ*) containing disruptions in chromosomal *IDP* loci [1]. Yeast transformants were grown at 30°C in rich YP medium (1% yeast extract, 2% Bacto-peptone) with 2% glycerol and 2% lactate as carbon sources, and cells were harvested at $OD_{600nm} \cong 5.0$. Purification of mouse and yeast histidine-tagged enzymes was conducted as previously described [1] and resulted in yields of ~2 mg of mamIDP1 and ~60 mg of mamIDP2/liter bacterial culture, and of ~0.3 mg of each yeast IDP isozyme/liter yeast culture. The mouse IDP1 enzyme was further purified using Affigel Blue column chromatography [20]. Protein concentrations were determined using calculated extinction coefficients [21]. Purified IDP enzymes were used for experimental determination of pI values using isoelectric focusing gels (pH 3-10, Invitrogen).

Enzyme assays for yeast isozymes were conducted as previously described [1], and assays of mouse IDP1 and IDP2 were conducted using triethanolamine buffers as previously described [22]. Saturation kinetic curves were conducted at pH values ranging from 5.5 to 9.0 for the decarboxylation reaction (with 0.5 mM NADP⁺ and increasing concentrations of D-isocitrate, or with 2.5 mM D-isocitrate and increasing NADP⁺ concentrations) and for the carboxylation reaction (with 0.25 mM NADPH and increasing α -ketoglutarate concentrations, or with 2.5 mM α -ketoglutarate and increasing NADPH concentrations).

Expression of mamIDP2 in yeast

The parental haploid yeast strain used for expression of mouse IDP2 (mamIDP2) was MMY011 (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100*) [23] and derivatives of this strain (*idp2 Δ* and *idp2 Δ idp3 Δ*) containing deletion/disruptions of the yeast *IDP2* gene with *URA3* (or *kanMX4*) [24] and of the *IDP3* gene with *URA3*. A heterologous *Schizosaccharomyces pombe HIS5* gene [25] was used to disrupt the *ZWF1* gene in yeast *idp2 Δ* and *idp2 Δ idp3 Δ* mutants. Expression was initially tested following integration of the mouse cDNA into yeast *IDP2* or *IDP3* chromosomal loci. However, expression of the mouse enzyme using the genomic yeast promoters produced levels five-to ten-fold lower than those of the authentic yeast isozymes. Therefore, the mouse IDP2 cDNA was subcloned using PCR into a single-copy centromeric yeast plasmid (pCM252, EUROSCARF) for expression of mamIDP2 using a tetracycline (doxycycline)-inducible promoter (*tetO7-rtTA*) [26]. Similarly, a plasmid was generated for expression of a mamIDP2 ^{Δ AKL} enzyme in yeast using PCR primers lacking codons for the Ala-Leu-Lys tripeptide peroxisomal targeting sequence of mouse IDP2. All constructs were verified by DNA sequence analysis.

Immunoblots were used to compare expression of mamIDP2 (and of mamIDP2 ^{Δ AKL}) in parental and mutant yeast strains with levels of expression of endogenous yeast IDP2 and IDP3. Transformant strains were routinely pre-grown in minimal medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.5) lacking tryptophan for plasmid selection and with 2% glycerol as the carbon source for 12 h. In initial tests of expression, strains were diluted to $OD_{600nm} = 0.3$ and grown to $OD_{600nm} \cong 2.0$ in rich YP medium with 0.1% oleate (plus 0.2% Tween 40 to improve solubility) as the carbon source and with concentrations of doxycycline ranging from 0 to 2.0 μ g/ml. No detrimental effects of growth were obtained with any concentration of doxycycline tested. Expression levels of mamIDP2 obtained with 0.8 μ g doxycycline/ml were comparable to endogenous levels of yeast IDP2 plus IDP3, and this

concentration was used in subsequent experiments. To maintain constant expression levels of mamIDP2 during extended cultivation, 0.4 µg doxycycline/ml culture was added at 12 h intervals.

Subcellular fractionation and immunoblot analyses

Organellar pellets and soluble 'cytosolic' fractions from yeast parental and transformant strains grown on YP medium with ethanol (2%) or 0.1% petroselinate (plus 0.2% Tween 40) as the carbon source were prepared by differential centrifugation of yeast cellular homogenates as previously described [27]. Protein concentrations were determined using the Bradford method [28].

Protein samples (~5 µg ea) of whole cellular extracts or of 'cytosolic' and organellar pellet fractions were electrophoresed using 10% polyacrylamide/sodium dodecylsulfate gels. Immunoblot analyses were conducted using an antiserum that recognizes both yeast IDP1 and IDP2 [7], and antisera specific for yeast IDP3 [6], for yeast MDH3 [29], for yeast β-actin (AbCam), and for mammalian IDP2 [30]. Proteins were detected using the enhanced chemiluminescence method, and densitometry was conducted using Scion Image software. Controls for quantitative analyses were samples of known concentrations of mouse IDP2 and yeast IDP isozymes purified as described above and electrophoresed with cellular protein samples. Quantitative analyses also involved subtraction for breakage of mitochondria or peroxisomal organelles based on levels of peroxisomal MDH3 and of mitochondrial yeast IDP1 observed in soluble cytosolic fractions. Little or no contamination of organellar pellet fractions (by cytosolic yeast IDP2 or β-actin) was observed.

Analyses of complementation and expression

Plasmids for tetracycline-inducible expression of mamIDP2 or of mamIDP2^{ΔAKL} were transformed into a yeast strain (*idp3Δ*) containing a chromosomal disruption of the *IDP3* gene and into a yeast strain also containing disruptions of the *IDP2* and *ZWF1* genes, constructed as described above. Transformants were pre-grown in selective medium as described above and diluted into rich YP medium containing petroselinate or oleate as the carbon source. Cell samples taken every 12 h were diluted and plated onto rich YP agar plates with 2% glucose to quantify viable cell numbers. Colonies were counted after three days of growth at 30°C.

Results

Expression and purification of IDP isozymes

To compare kinetic and physical properties of yeast and mammalian IDP isozymes, yeast *IDP1*, *IDP2*, and *IDP3* genes were expressed in a yeast mutant strain lacking the endogenous enzymes, and mouse *IDP1* and *IDP2* cDNAs were subcloned for expression in *E. coli* as described under Materials and Methods. Histidine codons were introduced onto the 5' ends of the coding regions for protein purification by Ni²⁺-NTA chromatography [1]. Samples of the purified proteins were electrophoresed to assess purity (Fig. 1) and, in experiments described below, were electrophoresed along with cellular protein samples for immunoblots to quantify levels of isozyme expression *in vivo*.

pH optima and pI values

To determine optimum pH values for kinetic evaluation, complete isocitrate saturation curves were conducted at various pH values ranging from 5.5 to 9.0. As illustrated in Fig. 2A, cytosolic yeast IDP2 (●) has a narrow pH range for optimal activity with a clear peak at pH 7.5. Peroxisomal yeast IDP3 (○) has a somewhat wider pH range for activity with >80% of the optimal $V_{\max}$ value obtained between pH 7.5 and pH 8.5. In contrast, the mouse mamIDP2

enzyme (▼) has a very broad pH range for optimal activity, with >80% of the optimal $V_{\max}$ value observed from pH 6.5 to pH 9.0. Thus, yeast peroxisomal IDP3 exhibits maximal catalytic capacity under basic conditions, whereas activity of yeast IDP2 is maximum at neutral pH. This is generally consistent with function of yeast IDP3 in peroxisomes with a reported luminal pH value of 8.2 [31] and of yeast IDP2 in the cytosol with reported pH values ranging from 7 to 7.5 [31,32]. Notably, mamIDP2, which functions in both compartments, exhibits little dependence on pH and would be expected to be equally active under both conditions.

For comparison, we also determined optimal pH ranges for yeast and mammalian mitochondrial enzymes (IDP1s), which function under basic environmental conditions (pH 8-8.5 for the mitochondrial matrix) [32]. As shown in Fig. 2B, both yeast (●) and mouse (○) mitochondrial enzymes exhibit maximum velocities for oxidation of isocitrate at basic pH values, with curves similar to that obtained for yeast IDP3. In evaluating these isocitrate saturation curves, we found that apparent substrate K_m values for all of the IDP enzymes were largely unchanged by differences in pH.

A comparison of measured and reported pI values for yeast and mouse IDP isozymes is shown in Table 1. The basic pI values for yeast IDP3 and for yeast or mouse IDP1 enzymes are consistent with respective localization of these enzymes in peroxisomes and in mitochondria. Yeast IDP2 has a slightly acidic pI value, consistent with localization in the neutral cytosolic compartment. However, mamIDP2 exhibits a pI that is intermediate between those exhibited by solely cytosolic or peroxisomal yeast enzymes, again suggesting a property that would facilitate function of this enzyme in both cellular compartments.

Comparison of kinetic properties

Kinetic parameters for the decarboxylation reaction were determined for yeast and mammalian IDP isozymes at optimal pH values (determined as described above). As shown in Table 2, yeast mitochondrial IDP1 and peroxisomal IDP3 enzymes exhibit similar kinetic properties, as do mouse IDP1 and IDP2 enzymes. The latter pair exhibit lower apparent K_m values for substrate and cofactor than do yeast IDP1 and IDP3. Yeast IDP2 exhibits a substantially higher K_m value for isocitrate than the other enzymes and, as previously reported [1], has a similar K_m value for α -ketoglutarate in the reverse carboxylation reaction. We examined kinetic parameters of the other isozymes in the carboxylation reaction (data not shown) and determined that yeast IDP2 is unique in this property, since the other yeast and mammalian enzymes have substantially lower K_m values for isocitrate than for α -ketoglutarate. We previously suggested that cytosolic yeast IDP2 may be required to function in both decarboxylation and carboxylation reactions to facilitate shuttling of isocitrate and α -ketoglutarate among various cellular compartments [1]. Current data suggest that the other isozymes would have less capacity for such bidirectional function *in vivo*.

Overall, these data show that the kinetic properties of mamIDP2 are more similar to those of yeast peroxisomal IDP3 than to those of yeast cytosolic IDP2.

Regulated expression and compartmentalization of mamIDP2 in yeast

To examine compartmentalization and function of mamIDP2 in yeast, we expressed both the authentic mouse protein and a form of the protein (mamIDP2^{ΔAKL}) lacking the peroxisomal targeting signal at levels approximately equivalent to those of endogenous yeast isozymes. For this, the full-length mamIDP2 cDNA and a cDNA lacking the three codons at the 3'-end of the coding region were subcloned into a yeast plasmid with a tetracycline (doxycycline)-inducible promoter. The parental yeast strain and various *IDP* gene disruption mutants were transformed with the plasmids, and expression was induced using a range of concentrations of doxycycline. Representative results are shown in Fig. 3 for an *idp2Δidp3Δ* mutant yeast strain

transformed with the plasmids and grown in YP oleate medium with doxycycline. For both transformants, expression of mamIDP2 and mamIDP2^{ΔAKL} is inducible with doxycycline, although there is a basal level of expression in the absence of the drug. For the *idp2Δidp3Δ* and other transformant strains, these initial experiments were conducted with oleate because a fatty acid carbon source results in maximum cellular levels of both endogenous yeast IDP2 and IDP3 when present [12]. Immunoblot analyses of various transformant strains were conducted with cellular extracts using antisera specific for yeast IDP isozymes and for the mamIDP2 isozyme, and amounts of these proteins in total cellular protein extracts were determined using parallel immunoblot analyses of the purified isozymes. Based on these quantitative analyses, doxycycline concentrations of 0.8 μg/ml were found to produce levels of mamIDP2 or mamIDP2^{ΔAKL} (~15 ng/μg cellular protein) approximately equal to those of endogenous yeast IDP2 plus IDP3 (~9 ng/μg cellular protein) in cells grown on rich YP medium with oleate as the carbon source. For experiments involving growth of the transformant strains beyond 24 h, it was necessary to supplement the medium with doxycycline at 12 h intervals to maintain these expression levels.

To examine compartmental localization in yeast, cellular fractionation was conducted using the parental strain expressing mamIDP2 or mamIDP2^{ΔAKL} following growth in rich YP medium with doxycycline and either ethanol or petroselinic acid as the carbon source. These carbon sources were chosen to examine any effect on localization based on a requirement for a peroxisomal IDP activity for yeast growth with petroselinic acid but not with ethanol [9,12]. Harvested cells were fractionated to produce an organellar pellet (mitochondria and peroxisomes) and a soluble 'cytosolic' fraction. Immunoblots were used to determine the relative levels of mamIDP2 or mamIDP2^{ΔAKL} in each cellular fraction. Representative results shown in Fig. 4A (top panel) demonstrate that mamIDP2 is present at similar levels in extracts from yeast cells grown on rich YP medium with either ethanol or petroselinic acid as the carbon source (lanes 1). Furthermore, mamIDP2 is found in both the cytosol (lanes 2) and in organellar pellet fractions (lanes 3). As shown in Fig. 4A (lower panel), mamIDP2^{ΔAKL} is also present at similar levels in extracts from yeast cells grown with the same carbon sources (lanes 1). However, in contrast to the authentic enzyme, mamIDP2^{ΔAKL} is found exclusively in the cytosolic fractions (lanes 2) and not in the organellar pellets (lanes 3). Thus, removal of the peroxisomal targeting signal of mamIDP2 results in apparently complete cytosolic localization of the enzyme in yeast cells. Similar patterns for expression and distribution of the mamIDP2 and mamIDP2^{ΔAKL} were observed in transformants of various yeast mutants (data not shown) used in experiments described below.

Controls in these experiments (Fig. 4A) were immunoblots for yeast peroxisomal MDH3, which allows quantification of the breakage of peroxisomes and contamination of the cytosolic fraction (e.g. lanes 2, with petroselinic acid as the carbon source), and for cytosolic β-actin, which confirms by its absence the purity of organellar pellet fractions (lanes 3). Furthermore, since the parental strain was used for expression of mamIDP2 and mamIDP2^{ΔAKL} enzymes in these particular experiments, we also analyzed the expression and distribution of endogenous yeast isozymes in the same samples. In the mamIDP2 transformant (Fig. 4B, top panel), levels of the yeast IDP1 and IDP2 enzymes were similar in extracts from cells grown with both carbon sources (lanes 1). Mitochondrial IDP1 is primarily localized in the organellar pellet fractions (lanes 3), and serves as a control for mitochondrial breakage (e.g. low levels in cytosolic fractions, lanes 2). Yeast IDP2 is found in the cytosolic fractions (lanes 2). In contrast, yeast peroxisomal IDP3 is not expressed with ethanol but is expressed with petroselinic acid as the carbon source (Fig. 4B, top panel), and is exclusively found in the organellar pellet fraction (lane 3, petroselinic acid). Very similar patterns for expression and localization of the yeast IDP isozymes were observed in the mamIDP2^{ΔAKL} transformant (Fig. 4B, lower panel) and in the untransformed parental strain (data not shown). These results indicate that expression of the

mammalian enzyme does not affect levels of expression or localization of the endogenous yeast enzymes.

Densitometry performed with purified proteins as standards was used to quantify levels of expression of yeast and mammalian IDP isozymes in multiple experiments similar to those shown in Fig. 4. Levels of yeast IDP2 and IDP3 isozymes together comprised 0.5-0.8% of total cellular protein in various strains grown with either ethanol or petroselinic acid as the carbon source, while the level of mamIDP2 or mamIDP2^{ΔAKL} comprised 0.6-1.7% of the cellular protein in similar strains. Thus, levels of the heterologous enzyme were comparable to those of endogenous yeast enzymes. The mamIDP2^{ΔAKL} enzyme was found only in cytosolic cellular fractions in all transformant strains and under all growth conditions. In contrast, the authentic mamIDP2 enzyme was distributed between cytosolic and organellar pellet fractions. Importantly, the cytosolic:organellar distribution of mamIDP2 was found to be ~2:1 (~4:2 ng mamIDP2/μg cellular protein) in cells grown with ethanol as a carbon source but ~1:2 (~6 ng mamIDP2/11 μg cellular protein) in cells grown with petroselinic acid as the carbon source. The slightly higher total cellular levels of mamIDP2 in petroselinic acid- versus ethanol-grown cells likely reflect differences in tetracycline-inducible expression with the different carbon sources. However, these results do suggest that the cellular localization of this enzyme may be influenced by the carbon source, with preferential peroxisomal localization under conditions requiring a functional IDP enzyme in that compartment, i.e. to provide NADPH for reduction of the double bond following C6 of petroselinic acid.

Functional compensation by mamIDP2 for loss of yeast IDP isozymes

To test the extent that mammalian IDP2 can function in different cellular compartments of yeast cells, the mamIDP2 enzyme was expressed in mutant strains with specific growth defects due to the absence of yeast peroxisomal IDP3 and/or cytosolic IDP2. Compensation for these growth defects due to expression of the mammalian enzyme was tested by assessing viable cell numbers with time of growth in rich YP medium with restrictive fatty acid carbon sources. As shown in Fig. 5, over a 48 h period of cultivation, cell numbers for the parental yeast strain (●) increase ~ten-fold with growth in YP oleic acid medium (A) and ~six-fold with growth in YP petroselinic acid medium (B). The first test strain lacking yeast IDP3 (*idp3Δ*, ▼) is able to grow at parental rates with oleic acid (A) but, with petroselinic acid (B) as a carbon source, cell numbers only double over a 48 h period because of incomplete β-oxidation of the latter fatty acid. Expression of mamIDP2 in the *idp3Δ* strain (▽) has no effect on growth with oleic acid (A) but restores near parental rates of growth with petroselinic acid (B) over the same time period. The second yeast test strain lacking IDP2, IDP3, and ZWF1 (*idp2Δidp3Δ zwf1Δ*) was used to simultaneously assess function of mamIDP2 in the cytosol and in peroxisomes. The *idp2Δidp3Δ zwf1Δ* strain (■, Fig. 5) does not grow and, in fact, loses viability in medium with either oleic acid (A) or petroselinic acid (B) as the carbon source due to the absence of cytosolic sources of NADPH (IDP2 and ZWF1) and subsequent accumulation of hydrogen peroxide from each round of β-oxidation [9,11]. Expression of mamIDP2 in this strain (□) was found to restore growth with both carbon sources (Fig. 5, A and B), indicating function of the mammalian enzyme in both the cytosol (to permit removal of hydrogen peroxide generated during β-oxidation of both fatty acids) and in peroxisomes (to provide the peroxisomal NADPH needed for growth with petroselinic acid). Thus, mamIDP2 expressed at levels comparable to those of yeast IDP2 and IDP3 enzymes in the parental strain can compensate for loss of either or both compartmentalized isozymes.

To further assess functional complementation for the yeast isozymes, we expressed the mamIDP2^{ΔAKL} enzyme in both yeast test strains (Fig. 6). In medium with oleic acid as the carbon source (A), not only is the low viability of the *idp2Δidp3Δ zwf1Δ* strain (■) reversed by expression of the mamIDP2^{ΔAKL} enzyme (□) but the transformant strain also grows well over

the 48 h period. In contrast, in medium with petroselinic acid as the carbon source (B), the low viability of the *idp2Δidp3Δ zwf1Δ* strain (■) is compensated by expression of the mamIDP2^{ΔAKL} enzyme (□), but growth is restored only to the level of that observed for the *idp3Δ* strain (▼). Expression of the mamIDP2^{ΔAKL} enzyme in the *idp3Δ* strain (▽) also failed to improve growth of this strain on medium with petroselinic acid (B) as the carbon source. These patterns of complementation are those expected for an enzyme that compensates for yeast cytosolic IDP2 but not for peroxisomal IDP3. This is in accordance with effects predicted for removal of the mamIDP2 peroxisomal targeting signal and complete localization of the mamIDP2^{ΔAKL} enzyme in the cytosol, as documented above.

Discussion

In this report, we demonstrate that mammalian cytosolic/peroxisomal NADP⁺-specific isocitrate dehydrogenase (mamIDP2) is also localized in both cellular compartments when expressed in yeast cells. Furthermore, when expressed at levels comparable to the endogenous yeast enzymes, mamIDP2 can compensate for the absence of yeast IDP2 by permitting growth of an *idp2Δidp3Δ zwf1Δ* yeast strain in medium with a fatty acid carbon source. Thus, the mammalian enzyme can produce sufficient NADPH for cytosolic antioxidant systems that detoxify the hydrogen peroxide produced during peroxisomal β-oxidation [9,11]. The mamIDP2 enzyme can also compensate for loss of yeast IDP3 by permitting growth of an *idp3Δ* yeast strain (and of the *idp2Δidp3Δ zwf1Δ* strain) on carbon sources like petroselinic acid, i.e. on fatty acids with a double bond following an even-numbered carbon atom. Thus, partial peroxisomal localization of mamIDP2 results in production of sufficient NADPH for reduction of such double bonds during β-oxidation [5,6].

In contrast, expression of the mamIDP2^{ΔAKL} enzyme in yeast at levels of the endogenous yeast eliminates peroxisomal localization. The cytosolic mamIDP2^{ΔAKL} enzyme, as might be predicted, can compensate for loss of yeast cytosolic IDP2 (e.g. for growth with oleate as the carbon source) but not for loss of peroxisomal IDP3 (e.g. for growth with petroselinic acid). We note that previous studies of complementation by the mammalian enzyme in yeast [12] suggested that an enzyme lacking the peroxisomal targeting signal could still localize to peroxisomes and functionally substitute for yeast IDP3. However, those studies utilized multicopy vectors, and subsequent high levels of the heterologous enzyme presumably overwhelmed the peroxisomal import system. Thus, studies of compartmentalization and function *in vivo* require near normal levels of expression of a catalytically competent heterologous enzyme as reported here.

An important outcome of the current study is evidence that, while the peroxisomal targeting sequence of mamIDP2 is necessary for peroxisomal localization in yeast cells (as it is in mammalian cells [13]), this sequence does not ensure such localization. In other words, a substantial proportion of the enzyme is localized in the cytosol despite the presence of the carboxyl-terminal tripeptide signal. This suggests that some inherent property of the enzyme, potentially a cytosolic retention element, may prevent complete peroxisomal import both in mammalian and in yeast cells. This property will be examined in future experiments using hybrid fusion proteins.

The yeast expression system described here will be appropriate for assessing environmental and cellular factors that influence the distribution of mamIDP2 between cytosolic and peroxisomal locations. For example, we have some preliminary evidence that growth with a carbon source (petroselinic acid) that requires a functional peroxisomal activity may produce an increase in the proportion of mamIDP2 localized in the peroxisomes relative to growth with a carbon source (ethanol) that does not require a peroxisomal activity. Also, while the dual compartmental localization of mamIDP2 is unusual, at least one other human antioxidant

enzyme (PMP20) is similarly located in the cytosol and in peroxisomes [33]. It will be of interest to determine if similar factors affect distribution of both enzymes in yeast, and known mutant strains with defects in peroxisomal import and function [34,35] can be applied to assess factors involved in the dual compartmental localization of these mammalian enzymes. Finally, while previous studies have demonstrated that levels of mamIDP2 vary in a tissue-specific manner [19] and are dramatically elevated under certain conditions, e.g. in lactating bovine mammary gland [36] and in rat ovary during gonadotropin-induced development [30], nothing is known about differential compartmental localization of the enzyme under these conditions. Thus, our studies will be extended to mammalian cell lines and tissues.

A limitation of the yeast expression system is that mamIDP2 likely fulfills many cellular functions, particularly in peroxisomes, that may not be applicable in yeast cells. For example, while mammalian hepatocyte peroxisomes function in the β -oxidation of very long-chain fatty acids, and NADPH would be needed to reposition double bonds during β -oxidation of some of these fatty acids [37,38], a peroxisomal source of NADPH is also required for steps in the biosynthesis of isoprenoids, ether phospholipids, and potentially cholesterol [39,40]. With respect to antioxidant functions of cytosolically localized mamID2, most long- and short-chain fatty acids are degraded by β -oxidation in mammalian cell mitochondria without concomitant production of hydrogen peroxide [10]. However, numerous mammalian peroxisomal oxidases produce hydrogen peroxide as a byproduct [37,41]. Thus, while specific processes and reactions may not be evolutionarily conserved, basic requirements for both peroxisomal and cytosolic sources of NADPH appear to be common in eukaryotic cells. An obvious necessity for coordinate function of cytosolic and peroxisomal isocitrate dehydrogenases in fulfilling their cellular roles is an isocitrate/ α -ketoglutarate shuttle system in the peroxisomal membrane to indirectly facilitate exchange of NADP(H). Such a system, a peroxisomal transporter for isocitrate and α -ketoglutarate, has recently been described for mammalian cells [18] and is postulated to exist in lower eukaryotic cells as well [42].

To our knowledge, the current report on kinetic properties is the first direct comparison of mammalian IDP1 and IDP2 enzymes from the same species. Despite some differences in pH optima and pI values, the kinetic properties of the mouse IDP1 and IDP2 enzymes are quite similar. The mammalian IDP1 enzyme has been extensively characterized [22,43,44], and our values for the mouse enzyme are similar to those reported for the purified recombinant human enzyme [44]. In contrast, although the crystal structures of both mamIDP1 [45] and mamIDP2 [46] have been reported, there have been fewer kinetic studies of the mamIDP2 enzyme. Our values for the mouse IDP2 enzyme are quite similar to those previously reported for the rat IDP2 enzyme [47]. However, they differ from those previously reported for the human enzyme [13]. The latter report suggested substantially higher K_m values for isocitrate and NADP^+ (76 μM and 112 μM , respectively) for the recombinant human enzyme than those we measured (~ 4 μM for both ligands) for the recombinant mouse IDP2. It seems unlikely that enzymes sharing identities of $>95\%$ would have such dissimilar properties. It is more likely that differences are due to affinity purification schemes (use of a maltose-binding fusion protein for the human enzyme *versus* use of a histidine tag for the mouse enzyme) or to storage methods prior to kinetic analyses (ammonium precipitation of the human enzyme *versus* short storage at 4°C for the mouse enzyme).

Our characterizations of purified yeast and mammalian IDPs are consistent with the concept that the enzymes have evolved for optimum function at least with respect to their cellular pH environments. The enzymes found in one cellular location (both mitochondrial IDPs, yeast peroxisomal IDP3, and yeast cytosolic IDP2) have pH optima for catalysis and pI values in accordance with the pH value in each cellular compartment. The mamIDP2 enzyme, on the other hand, has a very broad neutral to basic pH optimum for catalysis and a pI value intermediate to the pH values reported for the cytosol and peroxisomal matrix [31]. In

mammalian cells and in yeast cells, as shown in this study, the enzyme functions well in both cellular compartments. Interestingly, a previous study demonstrated that replacement of yeast cytosolic IDP2 with the yeast mitochondrial IDP1 or the peroxisomal IDP3 enzyme did not provide complementation for growth of an *idp2Δzwf1Δ* strain with a fatty acid carbon source [48]. This suggests that the mislocalized yeast isozymes may be compromised *in vivo*, perhaps due to suboptimum catalytic pH values or pI values for function in the cytosol. Clearly, it will be of interest to determine if yeast IDP2, which has a very narrow neutral pH optimum for catalysis and a slightly acidic pI value, can be localized to peroxisomes and if it can fulfill the function of peroxisomal IDP3.

The existence of NADP-specific isocitrate dehydrogenase activity in multiple cellular compartments appears to be a general theme in eukaryotic organisms. However, as with yeast and mammalian cells, differential localization is accomplished by different mechanisms. In addition to proteins analogous to the three genetically distinct isozymes present in yeast cells, plants also have a chloroplast enzyme [49]. In contrast, a single gene in *Aspergillus sp.* encodes mitochondrial, cytosolic, and peroxisomal enzymes [50,51]. In *A. nidulans*, two different transcriptional start sites are used to produce a longer transcript encoding a protein with an amino-terminal mitochondrial targeting sequence or a shorter transcript lacking that sequence [50]. The shorter transcript encodes a protein with a likely peroxisomal targeting signal (a carboxyl-terminal Ala-Arg-Leu tripeptide), and thus presumably is translated to produce both cytosolic and peroxisomal enzymes, as is the case for the non-mitochondrial mammalian isozyme. Our studies lead to a prediction that the genetically distinct enzymes have divergently evolved for function in their specific compartments, whereas enzymes like the *A. nidulans* IDP may exhibit intermediate physical properties to permit function in different cellular environments.

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Fig. 1.

Affinity purified IDP isozymes. Following expression and purification of His-tagged IDP isozymes, protein samples (~1.0 μg ea) were electrophoresed under denaturing conditions and stained with Coomassie blue. Yeast IDP1 (lane 1), IDP2 (lane 2), and IDP3 (lane 3) isozymes, and mouse IDP1 (lane 4) and IDP2 (lane 5) isozymes are homodimers with similar subunit molecular sizes (46-48 kDa).

Fig. 2.

Apparent $V_{\max}$ values for IDP isozymes as a function of pH. Full isocitrate saturation kinetic analyses were conducted at the indicated pH values using purified non-mitochondrial IDP isozymes (A: yeast IDP2, ●; yeast IDP3, ○; and mouse IDP2, ▼) or purified mitochondrial IDP isozymes (B: yeast IDP1, ●; and mouse IDP1, ○). Apparent $V_{\max}$ values were expressed relative to the maximum value obtained for each enzyme.

Fig. 3.

Doxycycline-inducible expression of mamIDP2 in yeast. An *idp2 Δ idp3 Δ* yeast mutant strain was transformed with plasmids containing the mouse *IDP2* (top panel) or *IDP2 Δ AKL* cDNA (bottom panel) following a tetracycline (doxycycline)-inducible promoter. The transformants were cultivated in rich YP medium with oleate as the carbon source and the indicated concentrations of doxycycline as described under Materials and Methods. Cellular protein extracts (~5.0 μ g ea) were electrophoresed for immunoblot analyses using an antiserum specific for mammalian IDP2 and, as controls, antiserum for yeast β -actin or for the peroxisomal isozyme of malate dehydrogenase (MDH3). Control lanes (ctl) contained extracts from the untransformed *idp2 Δ idp3 Δ* strain.

Fig. 4.

Compartmentalization of mamIDP2 in yeast. The compartmental distribution of mammalian IDP2 (A) and of endogenous yeast IDP isozymes (B) was examined in parental yeast strain transformants expressing mouse IDP2 (top panels) or mouse IDP2 Δ AKL (bottom panels). Transformants were cultivated in rich YP medium with either ethanol or petroselinate as the carbon source as indicated. Whole cellular homogenates (lanes 1) were subjected to differential centrifugation to obtain soluble 'cytosolic' fractions (lanes 2) and mitochondrial/peroxisomal organellar pellets (lanes 3). Protein samples (~5.0 μ g ea) were electrophoresed for immunoblot analyses using antisera specific for mammalian IDP2, for yeast peroxisomal MDH3, for yeast β -actin, for yeast IDP1 plus IDP2, and for yeast IDP3.

Fig. 5.

Tests of complementation by mamIDP2 for yeast IDP2 and IDP3. The parental yeast strain (●), an *idp3Δ* mutant strain (▼), an *idp2Δidp3Δzwf1Δ* mutant strain (■), and transformants of the latter two strains expressing mouse IDP2 (▽ and □, respectively) were grown on rich YP medium with oleate (A) or petroselinate (B) as the carbon source. At the indicated times, samples of the cultures were diluted and plated onto YP glucose agar plates to determine viable cell numbers. Numbers represent three independent measurements ($\pm$ SD).

Fig. 6. Tests of complementation by mamIDP2^{ΔAKL} for yeast IDP2 and IDP3. The parental yeast strain (●), an *idp3Δ* mutant strain (▼), an *idp2Δidp3Δzwf1Δ* mutant strain (■), and transformants of the latter two strains expressing mouse IDP2^{ΔAKL} (▽ and □, respectively) were grown on rich YP medium with oleate (A) or petroselinate (B) as the carbon source. At the indicated times, samples of the cultures were diluted and plated onto YP glucose agar plates to determine viable cell numbers. Numbers represent three independent measurements (±SD).

Table 1*pI* values for cellular isozymes of IDP

yeast IDP2	Non-mitochondrial yeast IDP3	mamIDP2	yeast IDP1	Mitochondrial mamIDP1
6.5 ^a (6.1) ^b	8.9 (10.0)	7.5 (6.5)	8.2 (9.5)	8.5 (8.9)

^a *pI* values were determined experimentally.

^b Predicted *pI* values based on protein sequences (obtained from the *Saccharomyces* Genome Database, <http://www.yeastgenome.org>, or calculated using Vector NTI suite 8 software) are shown in parentheses. Differences between experimental and predicted *pI* values may in part be due to histidine tags on the carboxyl termini of purified proteins.

Table 2

Kinetic parameters for cellular isozymes of IDP

	IDP1 ^a	Yeast IDP2 ^a	Mammalian	
			IDP3	IDP1 IDP2
$V_{\max}$ (U/mg)	65 (±17)	126 (±25)	48 (±1.3)	88 (±3.5) 63 (±0.9)
Isocitrate K_m (μM)	30 (±0)	220 (±30)	15 (±1.2)	5.8 (±0.5) 4.2 (±0.1)
NADP ⁺ K_m (μM)	30 (±0)	20 (±0)	10 (±1.6)	5.6 (±0.7) 3.7 (±0.1)

^aKinetic parameters were determined as described under Materials and Methods. Assays for each isozyme were conducted at optimal pH values determined as described in the text (pH 7.5 for yeast and mouse IDP2 enzymes, pH 8.0 for yeast and mouse IDP1 enzymes, and pH 8.5 for yeast IDP3).