Differential subcellular localization of endogenous and transfected soluble epoxide hydrolase in mammalian cells: evidence for isozyme variants

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Abstract Endogenous, constitutive soluble epoxide hydrolase in mice 3T3 cells was localized via immunofluorescence microscopy exclusively in peroxisomes, whereas transiently expressed mouse soluble epoxide hydrolase (from clofibrate-treated liver) accumulated only in the cytosol of 3T3 and HeLa cells. When the C-terminal Ile of mouse soluble epoxide hydrolase was mutated to generate a prototypic putative type 1 PTS (-SKI to -SKL), the enzyme targeted to peroxisomes. The possibility that soluble epoxide hydrolase-SKI was sorted slowly to peroxiosms from the cytosol was examined by stably expressing rat soluble epoxide hydrolase-SKI appended to the green fluorescent protein. Green fluorescent protein soluble epoxide hydrolase-SKI was strictly cytosolic, indicating that -SKI was not a temporally inefficient putative type 1 PTS. Import of soluble epoxide hydrolase-SKI into peroxisomes in plant cells revealed that the context of -SKI on soluble epoxide hydrolase was targeting permissible. These results show that the C-terminal -SKI is a non-functional putative type 1 PTS on soluble epoxide hydrolase and suggest the existence of distinct cytosolic and peroxisomal targeting variants of soluble epoxide hydrolase in mouse and rat.

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1. Introduction

Epoxide hydrolyases (EH: EC3.3.2.3) consist of a small family of at least five isoenzymes that catalyze the hydrolysis of a variety of toxic epoxides into their corresponding diols [1]. EH activities have been measured in at least two subcellular compartments of mammalian cells, including the cytosol (leukotrinene A4 EH and hepoxilin EH) and the endoplasmic reticulum (xenobiotic microsomal EH and cholesterol-5,6-EH) [1–4]. However, the precise compartmentalization of the fifth EH, soluble EH (sEH), remains controversial. Although initial studies suggested that sEH was localized within the mitochondria [5,6], later experiments employing isopycnic centrifugation revealed that sEH was actually localized within peroxisomes and the cytosol of mammalian cells [7,8]. Subsequent immunocytochemical analysis, however, suggested that

sEH was localized exclusively within the peroxisomes [9]. These authors speculated that the cytosolic compartmentalization of sEH reported in earlier biochemical studies was attributed to the fragmentation of the peroxisomes during tissue homogenization and/or organelle isolation procedures. More recently, however, Eriksson et al. [10] employed digitonin permeabilization studies of mice hepatocytes and demonstrated that the majority of sEH was localized in the cytosol with the remainder in peroxisomes. These results were supported by Arand et al. [11] who speculated that the dual localization of sEH was attributable to a functionally-impaired or functionally inefficient peroxisomal targeting signal (PTS) located at the C-terminus of the nascent enzyme.

Treatment of rodents with clofibrate, a hypolipidemic drug, causes the proliferation of peroxisomes and the induction of peroxisomal enzymes involved in fatty acid β-oxidation [12]. Clofibrate treatment, however, does not result in an increase in peroxisomal sEH-specific activity, whereas cytosolic sEH activity increases as a result of protein synthesis [13]. The differential effects of clofibrate administration on cytosolic and peroxisomal sEH has been considered in above studies dealing with the subcellular localization of sEH in mammalian cells, but a definitive relationship has not been elucidated.

In this study, we investigated the subcellular compartmentalization of sEH in cultured mammalian and plant cells using immunofluorescence microscopy. In addition, we tested whether the putative type 1 PTS (PTS1) on clofibrate-induced sEH, i.e. a C-terminal -Ser-Lys-Ile tripeptide (-SKI), was capable of targeting the enzyme (sEH-SKI) to cultured mammalian and plant peroxisomes. Our results show that the dual localization of sEH observed in mammalian cells is not a consequence of the enzymes C-terminal of -SKI functioning as an inefficient PTS1. Instead, transiently and stably expressed, clofibrate-induced sEH-SKI did not target to peroxisomes, but remained in the cytosol.

2. Materials and methods

2.1. Mammalian and plant cell cultures

The contact-inhibited NIH Swiss mouse embryo cell line NIH3T3 and the HeLa cell line (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 50 μg/ml gentamycin sulfate. V79 Chinese hamster lung fibroblast cells [14] were grown in DMEM with 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin. All mammalian cells were maintained in a humidified incubator at 37°C

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under 95% air and 5% CO2. Tobacco BY-2 (*Nicotiana tabacum* L. cv Bright Yellow 2) cell suspension cultures were grown in darkness at 25°C as described previously [15].

2.2. Plasmid construction

The murine sEH cDNA used in this study was isolated from a cDNA library constructed from mRNA isolated from liver tissue of mice that had been administered clofibrate in their diet [16]. DNA sequence within the *Nde*I and *Xho*I fragment of the mouse sEH cDNA (includes the entire ORF) was modified by site-directed mutagenesis as described [17] using the anti-sense primer 5′-TGGCTAA-AGCTTGGAGGTCAC-3′. This mutation resulted in a substitution of Leu for Ile at the C-terminus of mouse sEH (sEH-SKL). Mutant (-SKL) and wild-type (-SKI) mouse sEH clones were then excised from pKS+ (pBluescript, Stratagene) by digestion with *Nde*I and *Xho*I, blunt-ended with Klenow and subcloned into *Sma*I-digested pRTL2 [18] and *Sna*BI-digested pMT [19] for transient expression in tobacco or mammalian cells, respectively (Table 1).

For the construction of GFP-rat sEH expression plasmids, the GFP insert in the bacterial expression vector pBAD-GFP (Affymax) was PCR-amplified using Pfu DNA polymerase (Stratagene) and oligonucleotides 5′-TCGTGATCAGCCGCCATGGCTAGCAAAGGAGA-AGA-3′ and 5′-TCGTGATCAGATCTTTTGTATAGTTCATCCA-TGCC-3′. The resulting fragment was digested with *Bcl*I and ligated into the *Bam*HI-site of the mammalian expression vector pcDNA1/Amp (Invitrogen) to generate pcDNA1/Amp-GFP. Next, the *Xho*I/*Dra*I fragment from pRSET B-sEH1 encoding the entire ORF of clofibrate-induced rat sEH [20] was ligated into the *Sma*I/*Sal*I site of the cloning vector pSP64 Poly(A) (Promega). The resulting plasmid, pSP64-sEH, was digested with *Sst*I and *Bst*EII and religated in the presence of double-stranded oligonucleotides generated by hybridization of the two fragments 5′-GTGACCTCCAAGCNGTAGTAG-CT-3′ and 5′ACTACNGCTTGGAG-3′. Due to the degenerate code of the oligonucleotide at the site of mutagenesis, four different constructs were obtained, resulting in an exchange of the last codon of the rat sEH ORF. Finally, each of the different constructs were digested with *Eco*RI and partially digested with *Bgl*II. The resulting *Eco*RI/*Bgl*II fragments were inserted into the *Eco*RI/*Bgl*II sites of pcDNA1/Amp-GFP to generate each of the GFP-rat sEH fusion plasmids used in this study, i.e. GFP-rat sEH-SKI (wild-type), -SKL, -SKP, -SKQ, and -SKR (Table 1).

All DNA manipulations were verified by manual (T7 Sequencing Kit, Pharmacia) or TaqCycle automated sequencing (Applied Biosystems) with dideoxy terminators.

2.3. Transient transfections and transformations

NIH3T3 and HeLa cells were grown on coverslips to approximately 50% confluency, transfected with mouse sEH expression plasmids using LipofectACE reagent according to the manufacturer's instructions (Gibco BRL) and cultured in antibiotic-free DMEM without FCS for 24 h [19]. Cells were cultured for an additional 24 h in maintenance medium and then processed for indirect, immunofluorescence microscopy.

Tobacco BY-2 cells were transiently transformed by microprojectile bombardment with a helium biolistic particle delivery system according to the manufacturer's recommendations (Bio-Rad) [21]. Bombarded BY-2 cells were then left in unwrapped Petri dishes for 40 h and then processed for indirect, immunofluorescence microscopy.

2.4. Generation of V79 Chinese hamster stable cell lines

Stable transfection of V79 Chinese hamster cells was carried out by the calcium phosphate precipitation technique [22]. Briefly, approximately 7×10^5 cells were plated in 90 mm dishes and 24 h later, were co-transfected with either wild-type or modified GFP-rat sEH constructs and the selection plasmid pPUR (Clontech) that confers puromycin resistance. 24 h After transfection, cells were split 1:15 and maintained with selection medium (maintenance medium supplemented with 5 μg/ml puromycin). Resistant colonies appeared within 1–2 weeks and were subsequently isolated and maintained in selection medium until immunofluorescence microscopic analysis.

2.5. Immunofluorescence microscopy

NIH3T3 and HeLa cells on coverslips were washed three times in PBS, fixed in 3% (v/v) formaldehyde/PBS for 20 min and permeabilized with 1% Triton X-100/PBS for 15 min [19]. For experiments involving stable expression of GFP-rat sEH fusion proteins in V79 Chinese hamster cell lines, cells were grown on coverslips but not formaldehyde-fixed because fluorescence could be observed in living cells. Tobacco BY-2 cells were prepared for immunofluorescence microscopy as described previously [21]. For differential permeabilization experiments, NIH3T3, HeLa, and tobacco cells were permeabilized with digitonin (25 μg/ml) (Sigma) rather than Triton X-100 for 15 min to selectively permeabilize the plasma membrane [19,21].

Antibodies used for indirect immunofluorescence were as follows: rabbit anti-mouse sEH IgGs [23], rabbit anti-human erythrocyte catalase (Calbiochem), rabbit anti-rat catalase IgGs (kindly provided by Dariush Fahimi, University of Heidelberg), rabbit anti-cottonseed catalase IgGs [24], goat anti-rabbit BODIPY IgGs (Molecular Probes), goat anti-rabbit rhodamine IgGs and goat anti-rabbit Cy3
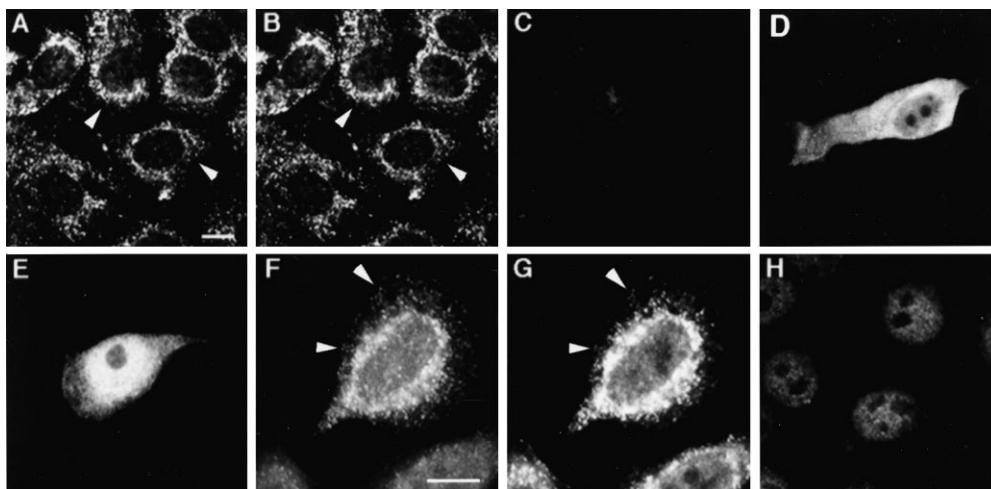

Fig. 1. Immunofluorescence localization of endogenous and wild-type or modified mouse sEH in cultured mammalian cells. Mice 3T3 (A–D) or HeLa (E–H) cells were grown on coverslips and either non-transfected or transfected with DNA encoding wild-type or modified mouse sEH. Cells were formaldehyde-fixed and then visualized by immunofluorescence microscopy. (A and B) Co-localization of endogenous sEH (A) and endogenous catalase (B) in peroxisomes of non-transfected mice 3T3 cells. Arrows indicate obvious co-localizations. (C) Representative field of non-transfected mice 3T3 cells with anti-mouse sEH IgGs omitted. (D) Cytosolic and peroxisomal localization of transiently expressed mouse sEH and endogenous sEH, respectively, in mice 3T3 cells. (E) Cytosolic localization of transiently expressed mouse sEH in HeLa cells. (F and G) Co-localization of transiently expressed mouse sEH-SKL (F) and endogenous catalase (G) in peroxisomes of HeLa cells. Arrows indicate obvious co-localizations. (H) Representative field of non-transfected HeLa cells with anti-mouse sEH IgGs omitted. Bar in (A) = 10 μm.

IgGs (Jackson ImmunoResearch Laboratories). Immunostained cells were mounted on glass slides in 90% (v/v) glycerol with *n*-propylgallate (Sigma) to prevent bleaching of fluorescence and viewed using either a Zeiss Axiovert 100 or a Mikrophot-FXA fluorescent microscope. In experiments involving expression of GFP-rat sEH fusion proteins, captured images were manipulated using Adobe Photoshop software (Adobe Systems).

3. Results

3.1. Localization of mouse sEH in mammalian cells

As the first step towards studying the subcellular localization of mammalian sEH, we examined endogenous sEH in mice 3T3 cells. Fig. 1 shows the results of double, indirect immunofluorescence staining of non-transfected mice 3T3 cells permeabilized with Triton X-100 and incubated with anti-mouse sEH IgGs (Fig. 1A) and anti-rat catalase IgGs (Fig. 1B). In all cells examined, sEH staining exhibited a particulate immunofluorescence pattern attributable to peroxisomal localization, as evidence by co-localization of sEH with endogenous peroxisomal catalase (Fig. 1A,B). No significant immunofluorescence staining attributable to the localization of endogenous sEH in the cytosol was detected (Fig. 1A). The fluorescence image attributable to peroxisomal sEH staining was not observed in cells when anti-mouse sEH IgGs were omitted (Fig. 1C). These results suggest that endogenous sEH is not cytosolic in murine cells but localized exclusively within peroxisomes.

The subcellular localization of transiently expressed mouse sEH in mouse 3T3 cells, however, was distinct from endogenous peroxisomal sEH. When mouse 3T3 cells were transfected with DNA encoding mouse sEH DNA and incubated with anti-mouse sEH IgGs, both a punctate and diffuse cytosolic immunofluorescence staining pattern was observed (Fig. 1D). These results were taken to indicate that endogenous and introduced sEH localized to two distinct subcellular compartments. That is, the punctate staining pattern was attributable to the localization of endogenous sEH in peroxisomes as shown in Fig. 1A and the diffuse staining pattern was the result of introduced mouse sEH localized in the cytosol. Because the immunodetection of both introduced and endogenous sEH in mouse cells hampered our ability to assess whether a portion of transfected mouse sEH was actually sorted from the cytosol to peroxisomes, we examined the localization of mouse sEH in HeLa cells. Preliminary experiments revealed that antibodies to mouse sEH did not recognize endogenous sEH in HeLa cells (data not shown). This is
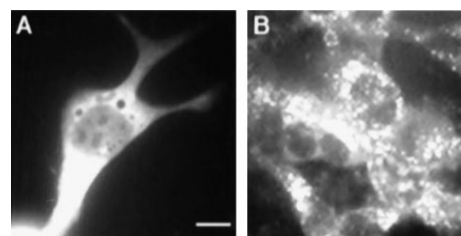

Fig. 2. Subcellular localization of stably-expressed GFP-rat sEH fusion proteins in V79 Chinese hamster cells. (A) Cytosolic localization of GFP-rat sEH-SKI. (B) Peroxisomal localization of rat sEH-GFP-SKL. Bar in (A) = 10 µm.

supported by previous results showing that this antiserum reacts much less strongly to the human sEH [23]. When HeLa cells were transfected with mouse sEH DNA and incubated with anti-mouse sEH IgGs, the immunofluorescence staining was diffuse (cytosolic) with no indication of peroxisomal labelling (Fig. 1E). This observation confirms the results illustrated in Fig. 1D, i.e. transiently expressed mouse sEH remains localized within the cytosol of mammalian cells. However, when the C-terminal Ile of mouse sEH was replaced with Leu thereby generating the prototypic PTS1 (i.e. -SKL), the transiently expressed enzyme (sEH-SKL) was convincingly directed to HeLa peroxisomes. Fig. 1F,G illustrate the co-localization of sEH-SKL with peroxisomal catalase. Authenticity of the targeting of mouse sEH-SKL to HeLa peroxisomes was reinforced in control experiments where anti-mouse sEH IgGs were omitted (Fig. 1H). In addition, differential detergent permeabilization experiments either with digitonin (permeabilizes only the plasma membrane) or Triton X-100 (permeabilizes all cellular membranes) showed that mouse sEH-SKL was actually imported into the peroxisome matrix rather than attached to the boundary membrane on the cytosolic side of the organelle (data not shown).

3.2. Stably expressed rat sEH is localized in the cytosol of mammalian cells

The cytosolic localization of transiently expressed wild-type mouse sEH-SKI showed above could be interpreted to show a temporally inefficient uptake of sEH-SKI into peroxisomes. That is, it is possible that a small proportion of introduced mouse sEH-SKI was imported into peroxisomes after 24 h and more would be imported with longer periods of time. Support for this premise comes from previous biochemical studies whereby the majority of mammalian sEH was reported

Table 1
Summary of subcellular localizations of various mouse sEH and GFP-rat sEH proteins in different cell types

Plasmids	Subcellular localization			
	NIH3T3	HeLa	V79	BY-2
pMT mouse sEH-SKI	C*	C		
pMT mouse sEH-SKL		P		
pcDNA1/AMP-GFP			C	
pcDNA1/AMP-GFP-rat sEH-SKI			C	
pcDNA1/AMP-GFP-rat sEH-SKP			C	
pcDNA1/AMP-GFP-rat sEH-SKQ			C	
pcDNA1/AMP-GFP-rat sEH-SKR			C	
pcDNA1/AMP-GFP-rat sEH-SKL			P	
pRTL2 mouse sEH-SKI				P
pRTL2 mouse sEH-SKL				P

(C) Cytosol, (P) Peroxisomes.
*Denotes that endogenous, peroxisomal sEH was detected with anti-mouse sEH IgGs.
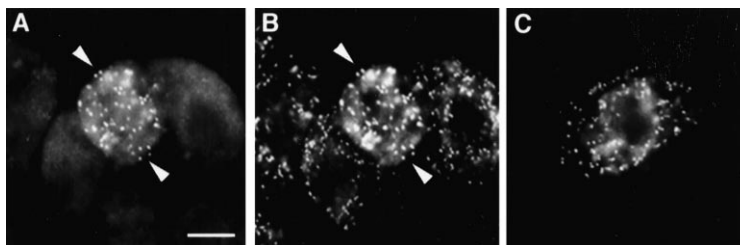
Fig. 3. Subcellular localization of transiently expressed wild-type or modified mouse sEH in tobacco BY-2 cells. Tobacco cells were biolistically bombarded with DNA encoding wild-type or modified mouse sEH, formaldehyde-fixed and then visualized by immunofluorescence microscopy. (A and B) Colocalization of mouse sEH-SKI (A) and catalase (B) in peroxisomes of a transiently transformed cell. Arrows indicate obvious co-localizations. (C) Peroxisomal localization of mouse sEH-SKL in a transiently transformed cell. Bar in (A) = 10 μm.

to reside in the cytosol with the remaining portion in peroxisomes [10,25]. To test this possibility, several V79 Chinese hamster cell lines stably expressing different GFP-rat sEH proteins were generated. As shown in Fig. 2A, stably expressed GFP-wild-type rat sEH-SKI was uniformly dispersed throughout the cytosol, not in a particulate pattern reminiscent of peroxisomal localization. Similar cytosolic localizations of GFP fluorescence were observed for GFP alone and for several other GFP constructs: GFP-rat sEH-SKP, -SKQ and -SKR (Table 1, photographs not shown). In constructs where Ile was replaced with Leu, however, the resulting fusion protein (GFP-rat sEH-SKL) was obviously sorted to peroxisomes (punctate fluorescence) without cytosolic staining (Fig. 2B). These observations support our interpretations from our results of transient transfection studies. That is, the C-terminal -SKI of sEH does not serve as a functional targeting signal for sorting sEH to mammalian peroxisomes even over an extended period of time.

3.3. Mouse sEH is sorted to peroxisomes in plant cells

We demonstrated previously in plant cells that a wide range of amino acid resides were functional within the PTS1 and that this range was considerably greater than those reported for the targeting of proteins to mammalian peroxisomes [21]. This knowledge prompted us to determine whether the C-terminal -SKI of mammalian sEH could indeed serve as a functional PTS1 in plant cells. DNA encoding mouse sEH-SKI was transiently expressed in tobacco BY-2 suspension culture cells. Fig. 3A,B show that mouse sEH-SKI was sorted to peroxisomes as evidenced by its co-localization with peroxisomal catalase. Results of the differential detergent permeabilization experiments revealed that transiently expressed mouse sEH actually was imported into the peroxisomes (data not shown). Fig. 3C shows that when Ile at the C-terminus of mouse sEH was replaced with Leu (sEH-SKL), peroxisomal targeting was preserved.

4. Discussion

The PTS1, a C-terminal uncleaved tripeptide -SKL motif, i.e. small basic hydrophobic residues, has been shown to be capable of directing proteins to the matrix of peroxisomes in evolutionary diverse organisms including mammals, yeast, trypanosomes and plants [26,27]. However, it has been experimentally shown that certain amino acid residues do not function within the tripeptide in various organisms despite the fact that they have characteristics similar to those defined by the prototypic SKL motif. For example, substitution of Ile for

Leu (both hydrophobic) at the −1 position of the PTS1 abolished targeting of proteins to mammalian peroxisomes [28], whereas Ile at the −1 position was functional for targeting proteins to yeast, trypanosome and plant peroxisomes [21,29,30]. Our working hypothesis, therefore, was that introduced wild-type mouse and rat sEH, both possessing a C-terminal -SKI [16,20], would accumulate in the cytosol and not target to peroxisomes in cultured mammalian cells. This hypothesis was not in accordance with interpretations made from previous studies where sEH was believed to be localized only in the peroxisomes [9], or in both peroxisomes and the cytosol [7,8,10,25]. Our results indicate that -SKI does not function as a PTS1, leading us to conclude that sEH-SKI is a cytosolic enzyme. This conclusion, however, must be reconciled with our immunofluorescence detection with anti-sEH IgGs of endogenous sEH exclusively within peroxisomes in mice 3T3 cells (Fig. 1A) and with numerous biochemical studies demonstrating the cytosolic and peroxisomal localization of sEH in renal and hepatic tissues [13].

A possible explanation for the observed dual localization of sEH in mammalian cells is that the transient expression system employed herein (lipofection) did not provide adequate time for introduced sEH-SKI to be redirected from the cytosol to peroxisomes. That is, the observed localization of sEH-SKI in the cytosol simply could be interpreted as a kinetic manifestation whereby the introduced enzyme was targeted slowly to peroxisomes. This premise comes from Arand et al. [11] who proposed that the C-terminal -SKI of mammalian sEH functions as an impaired (temporally inefficient) PTS1 due to the Ile at the −1 position. From our results with stably expressed GFP constructs, this does not seem to be a likely explanation. GFP-sEH-SKI (and other modified constructs) remained in the cytosol (Fig. 2A, Table 1), with no evidence of punctate staining, thus, time was not a limiting factor for -SKI-directed targeting in mammalian cells. The context of -SKI at the C-terminus of sEH also does not appear to be the reason for the lack of peroxisomal targeting. Transiently-introduced mouse sEH-SKI was imported into plant peroxisomes (Fig. 3A,B) and a substitution of Leu for Ile at the −1 position (-SKL-COOH) on mouse and rat sEH resulted in their import into peroxisomes in both plant (Fig. 3D) and mammalian cells (Figs. 1F and 2B). Taken together, these observations indicated that the inability of introduced sEH-SKI to target to mammalian peroxisomes was not the result of its C-terminal -SKI being a temporally inefficient PTS1, or being spatially inaccessible to targeting signal receptors. Instead, introduced sEH-SKI seems to be an isoform variant of endogenous peroxisomal sEH.

The evidence suggests that two immuno-related forms (cytosolic and peroxisomal) of sEH exist in mammalian cells. Both forms of sEH may be encoded by one gene and the resulting gene products modified by one or possibly more pre- and/or post-translational mechanisms, e.g. multiple transcription initiation, alternative splicing of a common transcript, or post-translational modifications. There are several documented examples in which a single gene encodes proteins that are post-translationally targeted to an organelle, or remain in the cytosol [31]. For example, Bulitta et al. [32] reported that a single gene encoded the cytosolic and peroxisomal forms of guinea pig catalase.

Another reasonable consideration is that the two immunorelated forms of sEH are the products of two genes. Administration of the hypolipidemic drug clofibrate in the diets of rodents causes an increase in cytosolic sEH activity in liver cells, whereas peroxisomal sEH activity appears to be unaltered [33–36]. Hollinshead and Meijer [9], on the other hand, showed that clofibrate treatment did not lead to an increase in the cytosolic form of sEH. Both the mouse and rat sEH cDNAs employed in this study were isolated from cDNA libraries constructed from mRNA isolated from liver tissue of animals that had been administered clofibrate in their diet [16,20]. It is possible, therefore, that the sEH-SKI cDNAs represent the clofibrate induced cytosolic form and not the endogenous, constitutive peroxisomal sEH that was detected in Fig. 1A. Both the constitutive and the induced forms of mouse sEH have previously been shown to be immunologically indistinguishable using our antibody [23]. Evidence supporting a model for a constitutive sEH being targeted to and imported into peroxisiomes comes from studies of an sEH cDNA isolated from a human liver library made from cells that were not treated with clofibrate [37]. The deduced amino acid sequence revealed that this enzyme possesses a C-terminal -SKM tripeptide. Although it is not yet known whether this constitutive form of human sEH is localized exclusively within peroxisomes, a C-terminal -SKM has been shown in numerous studies to direct import of proteins into mammalian cell peroxisomes in vivo [19,28,38] and in vitro [39].

This paper presents compelling evidence that the dual localization of sEH in rodents is not the consequence of a single form of the enzyme localized in both the cytosol and peroxisomes as a consequence of an inefficient PTS1 (-SKI-COOH). Instead, we suggest that two distinct, yet immunorelated, forms of sEH are localized in these two separate subcellular compartments.

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