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Protein Quaternary Structure and Expression Levels Contribute to Peroxisomal Targeting Sequence 1-Mediated Peroxisomal Import of Human Soluble Epoxide Hydrolase

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Abstract

The Peroxisomal Targeting Sequence 1 (PTS1) is a consensus tripeptide, (S/C/A)(K/R/H)(L/M), found on the C-terminus of most peroxisomal proteins. However, the only known mammalian protein containing a terminal methionine PTS1 (SKM), human soluble epoxide hydrolase (hsEH), shows both peroxisomal and cytosolic localization *in vivo*. Mechanisms regulating the subcellular localization of hsEH thus remain unclear. Here we utilized GFP-hsEH fusion constructs to study the peroxisomal targeting of hsEH in transiently and stably-transfected Chinese hamster ovary cells. Our results suggest that the peroxisomal import of hsEH is regulated by three factors. First, we show that SKM is required but not sufficient for peroxisomal import. Second, by manipulating protein expression levels, we show that SKM mediates peroxisomal import of wild type hsEH only when expression levels are high. Third, we show that amino acid modifications which decrease subunit oligomerization and presumably enhance accessibility of the SKM motif confer peroxisomal targeting even at low protein expression levels. We conclude that in hsEH, SKM is a necessary but inefficient and context dependent PTS1. Peroxisomal import occurs when expression levels are high or when the SKM motif is accessible. These results provide a mechanistic basis for understanding the cell- and tissue-specific localization of hsEH *in vivo*.

Keywords

peroxisomal targeting sequence; Pex5p; subcellular localization; soluble epoxide hydrolase; polymorphism

Introduction

Soluble epoxide hydrolase (sEH, EC3.3.2.3) is a homodimeric enzyme possessing both a C-terminal epoxide hydrolase activity and a recently discovered N-terminal phosphatase activity.^{1–3} Previous studies in animals and in *in vitro* models suggest that the sEH C-terminal domain plays a role in inflammation,^{4–6} blood pressure regulation^{7,8} and cardiac hypertrophy⁹ by regulating the levels of endogenous fatty acid epoxides. It has also been suggested that the sEH

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N-terminal domain affects cell signaling, apoptosis and cholesterol synthesis through dephosphorylation of isoprenoid phosphates.^{10,11}

However, the biological significance of sEH in humans is not fully understood, partly due to controversy regarding its subcellular localization. Although analysis of protein sequence revealed peroxisomal targeting sequences in human soluble epoxide hydrolase (hsEH), a recent evaluation of a set of human tissue arrays shows exclusively cytosolic localization of hsEH in most tissues. The only exceptions are liver and kidney. In these two tissues, hsEH expression levels are much higher and hsEH is localized in both peroxisomes and the cytosol.¹² The mechanisms responsible for this apparent dual localization of hsEH are not known, but understanding how localization is controlled may aid in our understanding of the function(s) of this enzyme *in vivo*.

There is evidence that the substrates for hsEH exist in both peroxisomes and the cytosol. Fatty acid epoxides, which are substrates of the sEH C-terminal hydrolase domain, exist in the cytosol;^{13–15} whereas isoprenoid phosphates, which are *in vitro* substrates of the sEH N-terminal phosphatase domain, may exist in peroxisomes and/or cytosol.^{16,17} Therefore the subcellular localization of hsEH is likely important in determining its biological function(s).

Most peroxisomal matrix proteins have a C-terminal tripeptide peroxisomal targeting sequence (PTS1), (S/C/A)(K/R/H)(L/M).^{18–20} However, a small number of matrix proteins use an N-terminal consensus sequence (PTS2), (R/K)(L/V/I)X₅(H/Q)(L/A).^{20,21} PTS1 and PTS2 proteins are imported into peroxisomes via recognition by their corresponding receptors, Pex5p and Pex7p.²² There are several unusual aspects to the targeting sequences in hsEH. First, it is the only known mammalian protein that contains SKM as a PTS1. Also, unlike most other peroxisomal matrix proteins, hsEH contains both a putative PTS1 (SKM, amino acids 553–555)²³ and a putative PTS2 (KINRPMLQA, amino acids 101–109).¹² It also contains a PTS-2 like sequence (RLMKGEITL, amino acids 52–60), which has a Thr instead of Gln or Ala in a typical PTS2. The functional significance of having more than one PTS motif in a single protein is unknown, but makes the mainly cytosolic localization of hsEH all the more surprising.

The SKM tripeptide has been previously reported to be a functional PTS1 based on mutagenesis of SKL to SKM²⁴ or by appending SKM to non-peroxisomal proteins,²⁵ both of which resulted in peroxisomal localization. However, the targeting of an authentic SKM-type protein has not been studied. Interestingly, SKM has been documented to have more than a 10-fold lower binding affinity to Pex5p *in vitro* compared to SKL, the most efficient PTS1, and thus is considered a less potent PTS1.²⁶ Nevertheless, whether the inefficient binding of SKM *in vitro* contributes to hsEH cytosolic localization *in vivo* has not been investigated.

In this report, we show that the SKM PTS1 is required for hsEH peroxisomal import while the putative PTS2 is not involved in this process. We also show that the SKM PTS1 is able to target hsEH to peroxisomes only when the protein is expressed at high levels. In addition, mutations which block hsEH dimerization result in peroxisomal import regardless of protein expression level. Our results suggest that the SKM PTS1 motif of hsEH provides context-dependent subcellular targeting, thus allowing a protein with a single PTS1 to be differentially targeted in different cell types.

Results

hsEH requires a canonical PTS1 but not a PTS2 for peroxisomal import

CHO-K1 cells have been extensively used as a model system for analysis of peroxisomal matrix protein import. Here we constructed plasmids encoding wild type (WT) or modified hsEH fused to the C-terminus of GFP, and expressed the fusion proteins in CHO-K1 cells. The GFP

vector (Fig. 1a) was used as a negative control. After 72 hours, the localization of the GFP fusion proteins was analyzed by confocal microscopy. GFP-WT hSEH showed diffuse fluorescence in the cytosol and punctate spots distributed throughout the periphery which co-localized with the peroxisomal marker RFP(SKL) (Fig. 1b). To test whether hSEH is imported into peroxisomes via a PTS1 and/or PTS2 pathway, we designed modified hSEH constructs for analysis. A hSEH C-terminal construct, which contains amino acids 218–555 and has only a PTS1 motif (GFP-hSEH CT), showed strong peroxisomal localization (i.e. punctate fluorescence and little diffuse cytosolic fluorescence) (Fig. 1c). On the other hand, a hSEH N-terminal construct, which contains amino acids 1–238 and has only putative PTS2 motifs (GFP-hSEH NT), showed exclusively cytosolic distribution (Fig. 1d). To further confirm the non-functionality of the PTS2 motifs in hSEH, the SKM-PTS1 in hSEH was replaced by SKV, a non-PTS1 sequence. GFP-WT hSEH(SKV) (Fig. 1e) showed the same distribution pattern as GFP, a cytosolic protein containing no peroxisomal targeting sequences. The peroxisomal distribution of GFP-PHYH (a known PTS2 protein²⁷) was used to confirm that CHO-K1 cells are competent for PTS2 mediated peroxisomal import (Fig. 1f). Both the GFP-WT hSEH(SKV) and GFP-hSEH NT results provided evidence for the necessity of a PTS1 pathway and non-involvement of a PTS2 pathway in peroxisomal import of hSEH. Enzyme assays using *r*SO²⁸ demonstrated that GFP-tagged hSEH was catalytically active (data not shown). In addition, immunoblot data showed that hSEH was not cleaved from the GFP in CHO-K1 cells, and therefore the intracellular distribution of green fluorescence indeed represented the subcellular localization of hSEH (Fig. 1g).

SKM is a less efficient PTS1 than SKL in WT hSEH

The dual localization of GFP-WT hSEH even at 72 hour post-transfection suggested that the SKM motif was capable of targeting peroxisomal import, albeit not very efficiently. According to *in vitro* studies using fluorescence anisotropy, the SKM tripeptide has a lower binding affinity to the PTS1 receptor Pex5p than SKL, but is more potent than SKI (the PTS1 in rat and mouse hSEH).^{26,29} In order to compare the peroxisomal import efficiency of the three PTS1s *in vivo*, GFP-WT hSEH was expressed in CHO-K1 cells with SKL, SKM or SKI as PTS1. At all three time points during the 72 hour time-course of transient expression, more GFP-WT hSEH(SKL) was found in peroxisomes compared to GFP-WT hSEH(SKM) and GFP-WT hSEH(SKI) (Fig. 2a), suggesting that SKL is more efficient in targeting hSEH into peroxisomes. This result was confirmed by quantifying peroxisomal to cytosolic fluorescence ratios (P/C fluorescence ratio, Fig. 2b). Surprisingly, although it bears the same PTS1, GFP-WT hSEH(SKL) was less efficiently imported into peroxisomes than was the peroxisomal marker RFP(SK) (Fig. 2b). This finding suggested that peroxisomal import is also regulated by factors other than the PTS. There was no significant difference between SKM and SKI in peroxisomal import efficiency at any of the three time points ($p=0.06$).

Peroxisomal import of hSEH is modulated by quaternary structure

Previous *in vitro* studies have shown that WT hSEH mainly exists as a homodimeric complex formed by salt bridges between Arg287 and Glu256 of the two monomers.^{1,30} Visual analysis of the crystal structure of Pex5p-C (the PTS1 binding domain of Pex5p,³¹) and hSEH³² suggested that binding of the hSEH SKM to receptor Pex5p might be sterically hindered by the adjacent C-terminal domain of the other monomer. To test this hypothesis, we investigated the temporal *in vivo* peroxisomal localization of WT hSEH compared to several naturally occurring hSEH polymorphisms. Previous work has shown that two of these polymorphisms, R287Q and R103C/R287Q (double mutant, DM), decrease the ability of hSEH to dimerize, whereas the other polymorphisms (K55R, R103C and C154Y) do not.³⁰ We found that the subcellular localization of hSEH was correlated with its ability to dimerize. Similar to WT hSEH, hSEH polymorphisms showing normal dimerization (K55R, R103C and C154Y) had little peroxisomal localization. In contrast, for polymorphisms associated with altered

oligomerization (R287Q and DM,^{1,30} and data not shown), peroxisomal import occurred at earlier time points and the proportion of hSEH within peroxisomes was significantly increased compared to that seen with WT hSEH (Fig. 3a–c). In addition, despite having a less efficient PTS1, the peroxisomal/cytosolic fluorescence ratio of R287Q and DM hSEH polymorphisms at 24 hour post-transfection (Fig. 3a) was similar to that of hSEH carrying the most potent PTS1, WT hSEH(SKL) (Fig. 2A, 3b–c). Surprisingly, at later time points the proportion of R287Q and DM hSEH polymorphisms inside peroxisomes (Fig. 3a) exceeded that seen with WT hSEH(SKL) (Fig. 2a,3b–c). In fact, there was no difference in peroxisomal/cytosolic fluorescence ratio between R287Q and DM hSEH polymorphisms and the peroxisomal marker RFP(SKL). These results showed that the peroxisomal import of hSEH can be increased by enhancing monomer formation, presumably by increasing the accessibility of the PTS1; and that a more favorable quaternary structure can overcome the peroxisomal targeting inefficiency of SKM.

Previous *in vitro* studies have shown that R287Q hSEH is less stable than the WT hSEH.³⁰ Therefore the above results showing that R287Q hSEH has a higher peroxisomal/cytosolic fluorescence ratio than WT hSEH could also be explained by a greater rate of cytosolic protein degradation. In order to examine the effect of protein degradation, we transfected CHO-K1 cells with GFP-WT hSEH or GFP-R287Q hSEH and tracked the change in cellular fluorescence throughout a 72-hour time course using flow cytometry. The geometric mean fluorescence of GFP-WT hSEH at 24 hour was set as 100% and fluorescence of other samples was compared to this value. The results (Fig. 4) showed that the cellular fluorescence (sum of peroxisomal fluorescence and cytosolic fluorescence) of the two proteins were similar throughout this time period and suggested that the higher peroxisomal/cytosolic fluorescence ratio of R287Q hSEH (Fig. 3c) is not due to differences in protein degradation. These data suggested that the higher peroxisomal/cytosolic fluorescence ratio of R287Q hSEH is due to its increased rate of peroxisomal import.

Protein expression level influences peroxisomal import of hSEH

As mentioned earlier, previous subcellular localization studies of endogenous hSEH in various human tissues have suggested that protein expression level might also influence the peroxisomal import of hSEH. We isolated a stable cell line expressing approximately 10-fold lower level of GFP-WT hSEH compared to transiently-transfected cells (Fig. 5a) and found that this cell line exhibited an exclusively cytosolic fluorescence (Fig. 5b). The same construct in transiently-transfected cells showed a clear punctuate localization (Fig. 1a). Similar results were obtained for K55R, R103C and C154Y hSEH polymorphisms (data not shown). Restoring high protein expression by retransfection of the stable cell line with GFP-WT hSEH resulted in a recovery of peroxisomal accumulation of green fluorescence (Fig. 5b), indicating that stable cells remained competent for peroxisomal import of GFP-WT hSEH. Interestingly, the R287Q and DM hSEH polymorphisms maintained a peroxisomal localization phenotype, even in stable cell lines (Fig. 5c, DM hSEH data not shown).

As further confirmation that protein expression level regulates the peroxisomal import of hSEH with SKM PTS1, transiently transfected cells expressing high or low levels of GFP-WT hSEH were isolated by flow cytometry and imaged separately. Of the cells with low protein expression, 90% showed cytosolic localization, while cells with high protein expression showed a substantial accumulation of GFP-WT hSEH within peroxisomes (Fig. 5d). Under the same conditions, all cells expressing GFP-R287Q hSEH showed significant peroxisomal accumulation of this protein independent of protein expression level (Fig. 5e). This result demonstrated that the peroxisomal import of hSEH with a more accessible PTS1 is not affected by protein expression level.

Discussion

The subcellular localization of hSEH has been an enigma for a number of years. It contains a putative PTS1 and two putative PTS2s and yet is found in the cytosol of most human tissues. It is, however, found in the cytosol and peroxisomes of human liver and kidney.¹² sEH also shows dual localization in rat liver and this was attributed to the rat PTS1 (SKI) as being an impaired PTS.³³ Since mammalian sEH contains two catalytic sites there has been speculation that subcellular localization may influence catalytic site functionality. Although PTS2 mediated peroxisomal import is not common, in most cases the PTS2 motif is located within the extreme N-terminus (the first 20 amino acids) and is exposed on the protein surface and therefore accessible by the PTS2 receptor. Here, we find that the hSEH PTS2 is completely non-functional. In order to exclude the possibility that appending GFP directly upstream of the putative PTS2 motif might block binding to the PTS2 receptor, we use phytyl-CoA 2-hydroxylase (PHYH) as a PTS2 positive control. This protein has a functional PTS2 at the extreme N-terminus (amino acids 9–16). The peroxisomal localization of GFP-PHYH suggests that appending GFP to the N-terminus does not necessarily block peroxisomal import via the PTS2 pathway. Thus, the cytosolic localization of PTS1-disabled hSEH indicates that neither the KINRPMLQA nor the RLMKGEITL motif is likely to act as a functional PTS2 sequence in hSEH and that the peroxisomal localization of hSEH is independent of a PTS2 pathway. Our results are consistent with a recent report suggesting that a more strict definition of the PTS2 consensus sequence, (R/K)(L/V/I/Q)X₂(L/V/I/H/Q)(L/S/G/A/K)X(H/Q)(L/A/F), may be more reliable in identifying possible PTS2 proteins.³³ Moreover, unlike other known PTS2 proteins, hSEH contains putative PTS2s much farther from the N-terminus (amino acids 52–60 and 101–109). Based on the crystal structure (not shown) these motifs are not exposed on the protein surface and are likely inaccessible by the PTS2 receptor. Our results therefore, indicate that PTS2 location and/or accessibility should also be considered as important factors.

Previous work has shown that substitution of SKL by SKM results in an 11-fold reduction in binding affinity *in vitro*.^{26,29} In our transient transfection study, although SKM caused a significant loss in GFP-hSEH peroxisomal import efficiency, the difference was much smaller than might be expected based on K_d values alone. Similarly, mutation of SKL to SKI has been shown to render the PTS1 binding affinity 48-fold lower *in vitro*, and a more than a 4-fold loss in binding affinity relative to SKM. However, SKI in GFP-hSEH appeared to be much more potent *in vivo*, possessing peroxisomal import efficiency similar to that of SKM. This result was confirmed using two hSEH polymorphisms, GFP-R287Q hSEH and GFP-DM hSEH, in which the difference in peroxisomal import efficiencies between SKM and SKI was not distinguishable (data not shown). While the *in vitro* studies used PTS1-containing peptides of only 5–8 amino acids in order to decrease the complexity of data analysis, and only measured binding to the receptor,²⁶ our *in vivo* results require peroxisomal import of intact hSEH. *In vivo*, protein structure adjacent to the PTS1 and the PTS1 import machinery likely plays an important role;^{20,34} amino acids immediately upstream of the extreme C-terminal PTS1 are implicated in PTS1 receptor recognition.^{35,36} Furthermore, upstream secondary or tertiary structure is also reported to affect import efficiency in plants.³⁷ Subsequent steps in the PTS1 pathway *in vivo* could compensate for the loss of efficiency in the recognition and binding step by Pex5p. For example, substitution of the consensus Leu by Ile in PTS1 (SKL to SKI) disrupts peroxisomal localization in other proteins.¹⁹ However, SKI in hSEH is still a functional, although less efficient, PTS1, suggesting that adjacent structure in hSEH can compensate for the inefficiency of SKI binding to Pex5p.

Although import of unfolded polypeptides across the peroxisomal membrane^{38,39} has been described, most peroxisomal matrix proteins are reported to be imported into peroxisomes in oligomeric forms and remain in the cytosol if oligomerization is disrupted.^{39–42} The only exception is pumpkin isocitrate lyase,⁴³ both monomeric and oligomeric forms of which are

substrates of the PTS1 receptor. However, it appears that hSEH is more efficiently translocated into peroxisomes in forms predominantly or exclusively monomeric. We used visual inspection of the crystal structures of Pex5p-C and the monomeric and dimeric forms of hSEH to explore possible binding orientations. Our data are consistent with the model shown in Fig 6. In this model, considerable steric hindrance prevents binding of Pex5p-C to hSEH. Of the many dimer orientations that we explored, none was found that allowed the necessary interaction between the active site of Pex5p-C and the last five amino acids in the hSEH dimer (Fig. 6a). Therefore we hypothesize that this protein likely follows a “monomer pathway” (Fig. 6b). We suggest that the peroxisomal import of hSEH is based on binding affinity as well as the accessibility of PTS1 to Pex5p. For WT hSEH, which is known to exist mainly as a dimer, SKM is an inefficient PTS1 because of low accessibility. Thus, in cells with low protein expression level (eg. stable transfection) WT hSEH remains cytosolic. However, we observe peroxisomal accumulation of this protein when we promote binding of hSEH to Pex5p by enhancing protein expression by about 10-fold (eg. in transient transfection or by retransfection of the stable cell line), or by increasing the binding affinity of PTS1 by replacing SKM with the more efficient SKL.

On the other hand, for the R287Q and DM polymorphisms which have disrupted dimer formation, and thus more accessible PTS1 motif, hSEH is imported into peroxisomes even when protein expression levels are low. Our proposed model (Fig. 6) is further supported by the fact that the R287Q and DM hSEH polymorphisms showed a significantly higher percentage of peroxisomal localization than hSEH(SKL) at 48 and 72 hour post-transfection even though they bear a less efficient PTS1 (Fig. 3d).

Here we provide evidence that PTS1-mediated peroxisomal import is controlled by several mechanisms: PTS binding affinity to its receptor, protein quaternary structure (which affects accessibility of the PTS) and protein expression level. Indeed, we show that the effect of PTS1 accessibility to Pex5p is significant for hSEH peroxisomal import, even overwhelming the contribution of the PTS1 motif. Our data provide the first known *in vivo* example where peroxisomal import can be enhanced by factors other than primary amino acid sequence.

Recent studies suggest that several proteins of the ER and mitochondria are also targeted into peroxisomes.^{44–46} For example, deletion of the mitochondrial targeting signal in rat liver serine:pyruvate aminotransferase resulted in a conformational change and targeting into peroxisomes even though this protein contains a supposedly non-PTS1 (SKF).⁴⁵ Based on our results, we would predict that SKF may also be a context dependent PTS1 and that a broader definition of the PTS1 may be warranted.

The constructs we used in this study, K55R, R103C, C154Y, R287Q and DM, are known human sEH polymorphisms.⁴⁷ Compared to WT hSEH, the R287Q and DM polymorphisms have previously been shown to have differences in enzyme activity and stability *in vitro*.^{30, 48} R287Q hSEH has been implicated in several human diseases including increased cholesterol levels in familial hypercholesterolemia, insulin resistance in Type II diabetes, coronary artery calcification in African-Americans and subclinical coronary atherosclerosis.^{49–54} However, the role of R287Q hSEH in these diseases is not clear. In this report we demonstrate that R287Q hSEH dramatically enhances peroxisomal targeting compared to WT hSEH. Our results suggest that subcellular translocation of hSEH from cytosol to peroxisomes could contribute to disease states that have been statistically associated with the R287Q genotype. To the best of our knowledge, hSEH is the first known example of a protein showing a change in cytosolic to peroxisomal import phenotype due to a naturally occurring polymorphism. Since *in vivo* substrates of the two hSEH domains may exist in separate cellular compartments, alteration of subcellular localization may significantly affect the biological function and clinical significance of hSEH.

Material and Methods

Plasmid Construction and Site-Directed Mutagenesis

Wild-type (WT) hSEH, the C terminal domain, and the K55R, R103C, C154Y, R287Q, and R103C/R287Q (Double mutant, DM) polymorphisms⁴⁷ were first cloned into *Bam*HI/*Xho*I sites of pFastBac and pCR-Script vectors as previously described.⁴⁷ The fragments were then subcloned into *Bgl*II/*Sal*I sites of pAcGFP-C1 vector (BD Bioscience) to produce green fluorescent protein (GFP) fusion constructs. When expressed in mammalian cells alone, GFP is a monomeric protein (BD Bioscience), and so does not affect dimerization of hSEH.

The N-terminal domain of WT hSEH was amplified using oligonucleotide primers 5'-TATAGGATCCATGACGCTGCGCGGCGC-3' and 5'-GCCGCTCGAGTCAGCTCATGTCACTTGGATTGC-3'. The amplified product was digested with *Bam*HI/*Xho*I, and inserted into *Bgl*II/*Sal*I sites of pAcGFP-C1.

The cDNA coding for human phytanoyl-CoA 2-hydroxylase was amplified from IRATp970D1133D (RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH) using oligonucleotide primers 5'-CGGCAGATCTATGGAGCAGCTTCG-3' and 5'-CGGGGTCGACGCTATTTCAAAGATTGG-3' and subcloned into *Bgl*II/*Sal*I sites of pAcGFP-C1.

Site-directed mutagenesis was used to change the C-terminal SKM to SKL (using oligonucleotide primers 5'-ACCGGTGGTCTCAAAGCTTTAGCTCGACGGTAC-3' and 5'-GTACCGTCGAGCTAAAGCTTTGAGACCACCGGT-3') and SKI (using oligonucleotide primers 5'-ACCGGTGGTCTCAAAGATCTAGCTCGACGGTAC-3' and 5'-GTACCGTCGAGCTAGATCTTTGAGACCACCGGT-3'). pDsRed2-Peroxi (BD Bioscience) encoding a fusion of *Discozyma Sp.* red fluorescent protein and a SKL PTS1 was used as a protein marker for peroxisomes.

All PCR products were completely sequenced to verify their identity (Seqwright, Inc.).

Cell culture, transient transfection and stable cell line construction

Chinese hamster ovary (CHO) K1 cells (ATCC) were maintained in F12K medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.5% antibiotics at 37°C in a humidified incubator under 95% air and 5% CO₂. When cells were grown to 90–100% confluency in T75 flasks (Fisher), they were seeded at 1.2×10^5 cells/well in 4-well chambered cover glasses (Nalgene), or at 7×10^5 cells/well in 6-well plates. After allowing the cells to attach for 24 hour, they were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol and cultivated in antibiotic-free, FBS-free F12K for 24 hour before changing to fresh maintenance medium. Co-transfection efficiency is around 40%.

To construct stable cell lines, cells were passaged at a 1:20 ratio into selection medium (maintenance medium supplemented with 1 mg/ml G418) 24 hour after transfection. GFP-positive resistant colonies were subsequently isolated and maintained in selection medium.

Confocal Microscopy

At 24, 48, and 72 hour post-transfection, cells were imaged with a 100x oil immersion objective on a Leica SP2 confocal microscope (Leica Microsystems, Inc.). GFP was excited at 488 nm and dsRED was excited at 543 nm using the sequential scan mode to eliminate channel crosstalk. Since most peroxisomes are located near the bottom of cells, all images were acquired approximately 1 μ m above the cover slip. Although this is not a direct measure of how much hSEH is in each compartment, it represents an appropriate relative measure of hSEH

peroxisomal localization. Single-plane confocal images of at least 15 independent cells were taken and analyzed in a blind manner for each time point of each sample. Each experiment was repeated at least three times to confirm reproducibility of results.

Images were analyzed with ImageJ analysis software (Rasband, W. S., ImageJ, U. S. National Institute of Health, <http://rsb.info.nih.gov/ij>, 1997–2006) by drawing regions of interest around individual cells, thresholding and measuring integrated pixel intensity of either the cytoplasmic region or the peroxisomal region. For each cell, the peroxisomal integrated pixel intensity was divided by cytosolic integrated pixel intensity, and recorded as P/C fluorescence ratio. ANOVA and Tukey's post hoc tests were used to analyze all data.

Immunoblot analysis and hsEH enzyme assay

At 48 hour post-transfection, cells from 6-well plates were harvested using 0.05% trypsin EDTA (Invitrogen). The cells were pelleted (200×g, 4°C, 10 minutes) and resuspended in 80 µl of suspension buffer (40 mM Tris, pH 8.0, 150 mM NaCl, 0.2 mM PMSF, 1 mM EDTA). hsEH activity was assayed using *trans*-[¹H]stilbene oxide (*t*SO) as previously described²⁸. After SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membrane (Millipore). Blots were blocked with 10% nonfat dry milk powder in tris-buffered-saline (TBS). Anti-GFP (Clontech), anti-hsEH, anti-β actin (Abcam) and the anti-mouse secondary antibody (Pierce) were diluted according to the manufacture's recommendation. Blots were washed with 0.05% Tween 20/TBS, followed by detection with ECL reagent (Millipore). Immunoreactive bands were visualized by Image Station 440CF and Molecular Imaging Software version 4.0.3 (Kodak).

Flow Cytometry

Cells expressing GFP-WT hsEH or GFP-R287Q hsEH were analyzed on a flow cytometer (FACSCalibur, BD Biosciences) using CellQuest. For degradation analysis, the geometric mean of fluorescence (fluorescence/cell) of the entire population was measured at 24, 48 and 72 hour post-transfection. Data from three separate experiments were combined. For cell sorting, the cytometer was gated to collect cells expressing either high or low levels of fluorescence. These cells were then cultured for 48 hour post-sort (a total of 72 hour post-transfection) before confocal imaging as described above. All images were taken under the same exposure setting.

Computational Model

Structures for docking were obtained from Dr. German A. Gomez (hsEH) or the PDB⁵⁵ (human Pex5, 1FCH⁵⁶). These were prepared by adding hydrogens and checking side-chain structures with the BioPolymer module of Sybyl7.3. The PTS binding regions were prepared using the BioPolymer module to alter the residues, and then set as a variety of conformations for docking experiments. All docking was carried out by hand and orientations were visually assessed for steric clashes and potential protein-ligand interactions. Attempts were also made to dock in an orientation that mimicked the co-crystallized ligand of Pex5p-C, and to minimize steric clashing of the two sub-units.

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Figure 1.

Peroxisomal import of hsEH requires PTS1. (a-f) GFP tagged WT and modified hsEH were expressed in CHO-K1 cells. All images were acquired 72 hour post-transfection. RFP-SKL was co-expressed in all cells as a peroxisomal marker. GFP (a), the GFP-hsEH N-terminal construct (d) and GFP-WT hsEH(SKV) (e) were localized exclusively in the cytosol. GFP-WT hsEH (b), the GFP-hsEH C-terminal construct (c) and GFP-PHYH (f) were co-localized with the peroxisomal marker RFP-SKL. (g) Lysates of CHO-K1 cells expressing GFP tagged WT and modified hsEH were analyzed by SDS-PAGE followed by immunoblotting with anti-GFP and anti-hsEH. 15µg total protein was loaded for each sample. Bar=10 µm

Figure 2.

SKM is less efficient in hEH peroxisomal import than SKL. (a) Subcellular localization of GFP-hsEH(SKL), GFP-hsEH(SKM) and GFP-hsEH(SKI) 24, 48, and 72 hour post-transfection. Peroxisomal localization was confirmed by coexpression with RFP-SKL (images not shown). (b) Quantitative analysis of peroxisomal/cytosolic fluorescence ratio of GFP-hsEH (SKL), GFP-hsEH(SKM) and GFP-hsEH(SKI) 24, 48, and 72 hour post-transfection. Data was analyzed using ImageJ (NIH) on at least 45 independent confocal pictures from three independent experiments. Significance was denoted by asterisks ($p < 0.001$). Error bars indicate mean $\pm$ s.e. Bar=10 μ m

Figure 3.

Peroxisomal import of GFP-hsEH is modulated by quaternary structure. (a) Subcellular localization of GFP tagged K55R, C154Y, R103C, R287Q and DM hseEH polymorphisms 24, 48, and 72 hour post-transfection. Peroxisomal localization was confirmed by coexpression with RFP-SKL (images not shown). (b) Percentage of total cells showing hseEH peroxisomal accumulation was recorded. About 100 cells were counted for each sample at each time point. Significance is denoted by asterisks ($p < 0.001$). (c) Quantitative analysis of peroxisomal/cytosolic fluorescence ratio at 24, 48, and 72 hour post-transfection. Data was analyzed using ImageJ on at least 15 independent confocal pictures from three independent experiments. Within each of the three time points (24, 48 and 72 hour), bars with different letters (a, b or c) are significantly different from one another ($p < 0.001$). Statistical differences between the three time points were not compared. Error bars indicate mean $\pm$ s.e. Bar=10 μ m

Figure 4.

Cellular fluorescence of GFP-WT hSEH was similar to that of GFP-R287Q hSEH. GFP-WT hSEH and GFP-R287Q hSEH were expressed in CHO-K1 cells. Fluorescence of 10,000 cells was then measured using a flow cytometer every 24 hours. The geometric mean of each sample was calculated using CellQuest. Fluorescence of GFP-WT hSEH at 24 hour post-transfection was set as 100%. Error bars indicate mean $\pm$ s.e.

Figure 5.

Peroxisomal import of GFP-WT hSEH is modulated by protein expression level. (a) Immunoblot analysis of GFP-WT hSEH expression in CHO-K1 cells using anti-GFP antibody. β actin levels were probed as a loading control. (b) Confocal microscopy of GFP-WT hSEH expressed in CHO-K1 cells after transient transfection (left), stable transfection (middle) and retransfection in a stable cell line (right). (c) Confocal microscopy of GFP-R287Q hSEH expression in stably-transfected CHO-K1 cells. (d) and (e), CHO-K1 cells transiently-transfected with GFP-WT hSEH (d) and GFP-R287Q hSEH (e) were sorted into low (M1) and high (M2)-fluorescence pools by fluorescence-activated cell sorting, plated, and analyzed by confocal microscopy. Error bars indicate mean $\pm$ s.e. Bar=10 μ m

Figure 6.

Schematic models for hSEH binding to Pex5p-C. (a) WT hSEH mainly exists as a dimer (green and yellow subunits), and its interaction with Pex5p-C (lavender) is inefficient due to an inaccessible SKM PTS1. The region of the non-binding dimeric subunit (yellow) that sterically hinders Pex5p-C binding is shown in teal. The two PTS1 tails (SKM and 4 upstream amino acids; the one that binds to Pex5p-C is in red and the one that hinders binding is in teal) are presented in different conformations due to their random nature. The teal PTS1 tail is shown fully extended to demonstrate size, while the red PTS1 tail is shown in a conformation that mimics a co-crystallized PTS1 with Pex5p-C.³¹ (b) One possible docking orientation of Pex5p-C (lavender) with the PTS1 tail (purple: with Pex5p-C bound, shown at the top; red: without Pex5p-C bound, shown at the bottom) of the hSEH monomer (green). The PTS1 tail is presented in an orientation that mimics a co-crystallized PTS1 with Pex5p-C. hSEH(SKL) also exists mainly as dimer, but monomer binding to Pex5p-C is enhanced due to a more efficient PTS1. R287Q and DM hSEH exist mainly as monomers due to disrupted dimer formation, and their binding to Pex5p-C is enhanced due to a more accessible PTS1. Images were created using PyMol.⁵⁷