The peroxisomal targeting sequence type 1 receptor, Pex5p, and the peroxisomal import efficiency of alanine:glyoxylate aminotransferase

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Unlike most organellar proteins, some peroxisomal proteins are often found in significant amounts in the cytosol. Such apparent import inefficiency is very marked in guinea pig (*Cavia porcellus*) hepatocytes in which the cytosolic levels of two peroxisomal proteins, catalase and alanine:glyoxylate aminotransferase (AGT), are much higher than those found in human (*Homo sapiens*) hepatocytes, for example. In an attempt to provide an explanation for this phenomenon, we have cloned the guinea pig *CpPEX5* gene, which encodes the peroxisomal targeting sequence type 1 (PTS1) import receptor Pex5p, and functionally compared it with its human homologue, *HsPex5p*. Our results showed the following: (1) *CpPEX5*, like its human homologue, encodes two splice variants differing by the presence or absence of an internal region of 37 amino acids; (2) both variants were expressed in all guinea pig tissues studied; (3) both variants were equally able to complement peroxisomal import of PTS1 proteins in micro-injected $\Delta pex5$ human fibroblasts; (4) *CpPex5p* was as efficient as *HsPex5p* in mediating the peroxisomal import of proteins possessing the consensus PTS1, Ser-Lys-Leu, but much less efficient in mediating the import of proteins possessing non-consensus PTS1s (i.e. Lys-Lys-Leu of human AGT and Ala-Asn-

Leu of human catalase); (5) reporter proteins with the consensus PTS1, Ser-Lys-Leu, inhibited the peroxisomal import of endogenous catalase, whereas AGT with the non-consensus Lys-Lys-Leu did not; (6) high concentrations of *HsPex5p*, but not *CpPex5p*, markedly inhibited the import of AGT, but not catalase or proteins ending in Ser-Lys-Leu; and (7) in the yeast two-hybrid system, AGT-Ser-Lys-Leu interacted with the tetratricopeptide repeat domain of *HsPex5p*, but AGT-Lys-Lys-Leu did not. In addition, AGT-Ser-Lys-Leu was targeted to peroxisomes in *Saccharomyces cerevisiae*, whereas AGT-Lys-Lys-Leu was not. These data suggest that the inefficient peroxisomal import of AGT and catalase in guinea pig cells is due to the inefficiency with which *CpPex5p* mediates the peroxisomal import of proteins containing non-consensus PTS1s. They also suggest that the non-consensus PTS1 of human AGT might interact with *HsPex5p* very differently compared with the consensus PTS1, Ser-Lys-Leu.

Key words: guinea pig Pex5p, peroxisomal protein import, protein targeting.

INTRODUCTION

The intermediary metabolic enzyme alanine:glyoxylate aminotransferase (AGT) is unusual in that its subcellular distribution varies between different animals. Depending on the particular species, AGT in hepatocytes can be peroxisomal, mitochondrial, or both peroxisomal and mitochondrial [1,2]. We have suggested previously that the main metabolic functions of AGT are different in each organelle, so that in mitochondria AGT fulfils a gluconeogenic role, whereas in peroxisomes its main role is one of glyoxylate detoxification [3]. In most mammals very little AGT is found in the cytosol. However, in the hepatocytes of a few species, such as the guinea pig (*Cavia porcellus*), significant amounts of AGT (approx. 40%) are cytosolic, the remainder being peroxisomal [2,4]. The metabolic role of cytosolic AGT is unknown. Although a number of other peroxisomal proteins, such as D-amino-acid oxidase and uricase, are not found to any significant extent in the cytosol of guinea pig hepatocytes, the classical peroxisomal marker enzyme catalase is almost all cytosolic (approx. 89%) [4–7].

Most peroxisomal matrix proteins are targeted by C-terminal tripeptides based on the peroxisomal targeting sequence type 1

(PTS1) consensus motif Ser/Ala/Cys-Lys/Arg/His-Leu/Met [8,9]. Their import is mediated by interaction with the tetratricopeptide repeat (TPR) region of the PTS1 import receptor Pex5p [10,11]. AGT is also imported into peroxisomes via the PTS1 pathway [12], but unlike most other peroxisomal proteins its C-terminal tripeptides never get closer than a two-out-of-three match with the conservative consensus PTS1. For example, the C-terminal tripeptides of guinea pig and human (*Homo sapiens*) AGT are His-Arg-Leu and Lys-Lys-Leu respectively [4,13]. The unusual nature of the AGT PTS1 is reinforced by the finding that although necessary for the peroxisomal targeting of AGT, it is insufficient to target a range of reporter proteins [12,14]. In all mammals studied so far, AGT is encoded by a single gene [4,13,15–17] and, in the case of the guinea pig and human AGTs, all gene products would be expected to possess a PTS1, albeit one that is atypical. Therefore it is not immediately obvious why the peroxisomal import of AGT should appear to be much less efficient in the guinea pig than it is in the human, for example. Our previous studies [4] showed that human AGT was also inefficiently targeted to peroxisomes when expressed in guinea pig cells, whereas both human and guinea pig AGT were targeted much more efficiently when expressed in human cells.

Abbreviations used: AGT, alanine:glyoxylate aminotransferase; GFP, green fluorescent protein; PTS1, peroxisomal targeting sequence type 1; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TPR, tetratricopeptide repeat.

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When the C-terminal tripeptide of human AGT was mutated to the consensus PTS1 (i.e. Ser-Lys-Leu), import efficiency in both human and guinea pig cell lines was markedly improved. The tentative conclusion drawn from these studies was that the inefficient peroxisomal import of AGT (either human or guinea pig) in guinea pig cells was due to the inefficiency with which the guinea pig peroxisomal import pathway was able to mediate the peroxisomal import of proteins containing non-consensus PTS1s, such as His-Arg-Leu and Lys-Lys-Leu [4].

In an attempt to further define the nature of this peroxisomal targeting inefficiency, we have cloned and functionally characterized the guinea pig *CpPEX5* gene, which encodes the first component of the peroxisomal protein import pathway (i.e. the PTS1 import receptor Pex5p). We have measured semi-quantitatively the ability of *CpPex5p* and its human orthologue *HsPex5p* to mediate the peroxisomal import of various endogenous and reporter proteins in microinjected human fibroblasts. Our results indicate that the inefficient peroxisomal import of AGT in guinea pig cells results from the inefficiency with which *CpPex5p* mediates the import of proteins possessing non-consensus PTS1s. In addition, we provide evidence which suggests that non-consensus PTS1s might functionally interact with Pex5p rather differently compared with consensus PTS1s.

EXPERIMENTAL

Cloning the *PEX5* gene

Reverse transcriptase (RT) PCR

Guinea pig liver cDNA was obtained by reverse transcription of guinea pig liver total RNA using an oligo-dT primer. Two primers, P1 and P2, were designed based on the published *HsPEX5* nucleotide sequence (database accession number X84899) and mouse (*Mus musculus*) *MmPEX5* expressed sequence tags (MMA08738 and MM1159158; see Table 1 for details of all the primers used in the present study, and Figure 1 for an overall view of the cloning strategy). PCR amplification of the cDNA using these primers produced a 698 bp fragment, which was gel purified and blunt-end cloned into the *EcoRV* site of pBluescript, to give pGPX5-1. PCR amplification of guinea pig liver cDNA using primer P3 (based on the *HsPEX5* nucleotide sequence) and primer P4 (based on the *CpPEX5* nucleotide sequence from pGPX5-1) gave two products; the smaller product

Figure 1 *CpPEX5* cloning strategy

The uppermost horizontal line represents the 2009 bp composite *CpPEX5* nucleotide sequence. The triplets corresponding to the translation initiation codon (ATG) and the stop codon (TGA) are shown. The (+)-strand (P8, P3, P1 and P5) and the (–)-strand PCR primers (P7, P6, P4, P2 and P9) are indicated. Clones pGPX5-3 and pGPX5-4 have a deletion of 111 bp (indicated by Δ) compared with clones pGPX5-4 and pGPX5-7 respectively. The broken line at the 3' end of the 5'-RACE clone (pGPX5-6) represents the sequence amplified in the first round of PCR that was not amplified by the second round semi-nested PCR. The anchor primers used to amplify the RACE products are not shown in this diagram.

was 1.257 kb, while the larger product was 111 bp longer. Both PCR products were gel purified and cloned into the pGEM-T vector (Promega), to give clones pGPX5-3 and pGPX5-4 respectively.

5'- and 3'-Rapid amplification of cDNA ends (RACE)

For 3'-RACE, guinea pig liver total RNA was reverse transcribed using an oligo(dT) anchor primer. PCR amplification of the cDNA was carried out using the PCR anchor primer and a guinea-pig-specific PEX5 primer, P5. In this PCR reaction the annealing temperature was increased from 55 °C to 60 °C. Analysis by gel electrophoresis showed a PCR product of approx. 1.4 kb. This band was gel purified and cloned into the pGEM-T vector, to give pGPX5-5. For 5'-RACE, reverse transcription was carried out on guinea pig liver total RNA using the *CpPEX5* primer P6 and the product was polyadenylated with terminal transferase. The first round of PCR amplification was performed

Table 1 Description of the PEX5 oligonucleotide primers used in the present study

The nucleotide numbering system refers to that used in the guinea pig sequence with nucleotide number 1 being 29 nucleotides upstream of the translation initiation codon. The underlining indicates restriction sites for *Clal* (continuous underlining) and *SaI* (broken underlining).

Primer	Sequence	Regions of <i>CpPEX5</i> to which primers map	Strand
P1	AACCGGACAGCACTGATGGCN	1236–1256	(+)
P2	CAGGCCAAACATAGCTAGGAN	1943–1923	(–)
P3	ATGGCAATGCGGAGCTGGTN	30–50	(+)
P4	TGCAGGGACTCGTTGGTGAAG	1288–1268	(–)
P5	TCGCTATAACTTGGGCATCAG	1706–1726	(+)
P6	GTTCAATCTCCTGCATCTCTG	315–295	(–)
P7	TCCTAAAGGTCTGAGGGGCAC	279–259	(–)
P8	CTGTGCCGGTCAGAGAGGTTG	1–21	(+)
P9	CACATCCAGGGCAGAGACGC	2009–1990	(–)
P10	CTGCACAGCTGCCTCGAAC	1121–1103	(–)
P11	GATGCTGTGGATGTGACTCAG	408–428	(+)
P12	GCTGAGGGTGATCACGCAG	1044–1026	(–)
Oligo d(T)-anchor	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTT		
PCR-anchor	GACCACGCGTATCGATGTCGAC		

using an oligo(dT) anchor primer and the P6 primer. A second round of PCR was then carried out on 5% of the first round product using the PCR anchor primer and a nested *CpPEX5* primer, P7. A product of approx. 350 bp was gel purified and cloned into the pGEM-T vector to give pGPX5-6.

RT-PCR to generate *CpPEX5* full-length cDNA

Guinea pig liver cDNA was obtained by reverse transcription of guinea pig liver total RNA (5 µg) using an oligo(dT) primer. PCR amplification of the cDNA was carried out using primers P8 and P9. Two products of expected size (2.0 kb and 1.9 kb) were gel purified and cloned into the pGEM-T vector to give pGPX5-7 and pGPX5-8 respectively.

Tissue-specific expression of PEX5 mRNA

Fresh samples of a number of guinea pig tissues (adipose tissue, adrenal gland, brain, heart, kidney, liver, lung, muscle, ovaries, skin, small intestine, spinal cord and spleen) were snap frozen in liquid nitrogen. The frozen tissues (approx. 30 mg) were ground to a fine powder and total RNA was extracted using the RNeasy Miniprep kit (Qiagen). Using 1–10 µl of purified total RNA, first-strand cDNA was synthesized by reverse transcription using reverse-strand primer P10. cDNA was amplified by PCR using primers P11 and P12. The PCR reaction mixture (20%) was loaded on to a 1% (w/v) agarose gel.

Mammalian expression plasmids

For expression in human fibroblasts, all constructs were cloned into pcDNA3 (Invitrogen). Expression plasmids encoding normal human AGT with a C-terminus of Lys-Lys-Leu (AGT-KKL) and an artificial AGT with a C-terminus of Ser-Lys-Leu (AGT-SKL) (originally called AGT α and AGT α -SKL respectively) were made as described previously [12]. Green fluorescent protein (GFP)-SKL, which consisted of the c-Myc epitope fused to the N-terminus and Ser-Lys-Leu fused to the C-terminus of a fluorescently optimized GFP variant (described previously as GFPmut2 [18]) was a gift from Dr Tom Chappell (MRC Laboratory for Molecular Cell Biology, University College London, U.K.). GFP was made from GFP-SKL by replacing the C-terminal SKL with LE. The long and short isoforms of *CpPEX5* cDNA were derived from pGPX5-7 and pGPX5-8 respectively (see above). The long and short isoforms of *HsPEX5* cDNA (GD106 and GD102 respectively) were a gift from Dr Stephen Gould (Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, U.S.A.).

Tissue culture, microinjection and immunofluorescence microscopy

The cells used in this study were either normal human fibroblasts (155BR) or $\Delta pex5$ human fibroblasts obtained from a patient with neonatal adrenoleukodystrophy (AAL85AD) [12,19], previously shown to contain a knockout mutation in the *PEX5* gene [20]. Cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F10 supplemented with 10% (v/v) foetal calf serum (Gibco BRL) at 37 °C under 5% CO₂. Cells (1×10^4) were seeded on to 13 mm diameter glass microscope coverslips 24 h prior to microinjection. Expression plasmids (see above) were injected into the nuclei of at least 100 cells at various concentrations in reverse PBS (4 mM Na₂HPO₄, 1 mM KH₂PO₄ and 140 mM KCl, pH 7.3), using an Eppendorf microinjector. Following injection the cells were incubated at 37 °C for 18–24 h and processed for indirect immunofluorescence microscopy.

The cells were washed in PBS (0.8% NaCl, 0.02% KCl and 0.11% Na₂HPO₄, pH 7.2) and fixed in freshly prepared 3% (w/v) paraformaldehyde for 15 min at room temperature, followed by permeabilization with 1% (w/v) Triton X-100 for 15 min at room temperature. The cells were then processed for double- or single-label immunofluorescence using various combinations of rabbit anti-(human AGT) and guinea pig anti-(human catalase) polyclonal sera.

AGT and endogenous catalase (the latter acting as a peroxisomal marker) were visualized with FITC-conjugated goat anti-(rabbit IgG) (Sigma) or biotinylated goat anti-(guinea pig IgG) (Vector Laboratories, Peterborough, U.K.) followed by avidin-labelled Texas Red (Vector Laboratories). All incubations were performed at room temperature for 15 min in PBS containing 3% (w/v) BSA. The cells were washed extensively in PBS after each incubation. GFP was visualized by observing its autofluorescence. The coverslips were mounted on to glass slides using Mowiol (Harlow Chemical Co. Ltd., Harlow, Essex, U.K.) containing diazabicyclo[2.2.2]octane (Sigma). The fluorescent staining pattern was visualized in a Bio-Rad MRC1000 confocal laser-scanning microscope.

Between 6 and 62 microinjected cells (mean = 26) were identified for each condition on the basis of reporter-protein expression, and were scored with respect to whether the fluorescence-labelling pattern was diffuse or punctate (including partly punctate and partly diffuse). The results showed that there was no obvious difference between the short and long *PEX5* isoforms for either human or guinea pig, although there were differences between *HsPEX5* and *CpPEX5*, the different *PEX5* cDNA concentrations and the different reporter constructs. In an attempt to obtain a more reliable result (i.e. to increase the number of cells counted for each condition) the data for the long and short isoforms were combined. As a result, for each condition distinguished in these experiments (i.e. combining the long and short results), between 25 and 109 cells (mean = 53) were counted.

Two-hybrid analysis

pGBT-AGT-KKL and pGBT-AGT-SKL were constructed by cloning the entire coding regions of AGT-KKL and AGT-SKL respectively into pGBT9 (Clontech Laboratories, Palo Alto, CA, U.S.A.) to create in-frame fusions between the GAL4 DNA-binding domain and AGT. These plasmids were used as partners in the two-hybrid system with pGAD-*ScPEX5* (originally pGAD424-*pas10*) and pGAD-*HsPEX5* (originally pPTS1-BP) as previously described [21]. These latter clones encode the yeast and human (TPR domain only) PTS1-receptor genes cloned into pGAD10 and pGAD424 (Clontech Laboratories), respectively, to create in-frame fusions with the GAL4 transactivation domain. The host strain was SFY526 (*MAT α* , *his3-200*, *ade2-101*, *lys2-801*, *trp1 901*, *leu2-3, 112*, *can1*, *gal4-542*, *gal80-538*) [22]. For β -galactosidase expression assays, the transformants were grown to mid-exponential phase in SD medium [0.67% yeast nitrogen base and 2% (w/v) dextrose] supplemented with appropriate amino acids prior to glass-bead homogenization, SDS/PAGE and immunoblotting with monoclonal anti-(β -galactosidase), at a dilution of 1:10000, as previously described [21].

Yeast expression plasmids

The coding region of human AGT-KKL was amplified using primers P13 (5'-CGCGACTAGTATGGCCTCTCACAAGC-3') and P14 (5'-CGCGAAGCTTTCACAGCTTCTTCTTGGG-GCAG-3'), or primers P13 and P15 (5'-CGCGAAGCTTTCAC-AGCTTAGACTTGGGGCAG-3'). The (+)-strand primer P13 introduces a *SpeI* site (underlined) directly preceding the start

codon, and the (–)-strand primers P14 and P15 introduce a *HindIII* site (underlined) after the stop codon. P15 is a mutagenic primer which changes the natural C-terminal KKL of AGT to SKL (marked in bold). The GFP insert in the plasmid yGFP-C-FUS [23] was removed by *SalI* digestion to give pMET25 (which contains the MET25 promoter and the *URA3* gene). The P13/P14 and P13/P15 amplification products of AGT-KKL and AGT-SKL, respectively, were digested with *SpeI* and *HindIII*, and cloned into pMET25 to give yAGT-KKL and yAGT-SKL. *Saccharomyces cerevisiae* strain SUB61 (*MAT α* , *lys2-80*, *leu2-3*, *2-122*, *ura3-52*, *his3-200*, *trp1-1*) was transformed with yAGT-KKL and yAGT-SKL and selected for its ability to grow in uracil-free medium.

Immunoelectron microscopic analysis of AGT targeting in *S. cerevisiae*

Cells were grown to mid-exponential density in SD medium containing glucose, and then transferred to medium deficient in methionine, to induce AGT synthesis, and containing oleate (0.1%) and Tween 40 (0.25%) to induce peroxisome proliferation. After 18 h the cells were pelleted by spinning at 10000 g for 2 min. They were then fixed in 2% (w/v) glutaraldehyde/0.1 M sodium cacodylate buffer (pH 7.2) for 4 h at room temperature. Following extensive washing in buffer, free aldehyde groups were quenched with 0.5 M NH_4Cl in cacodylate buffer. After further washing in buffer, the fixed cells were embedded in 2% low melting point agarose, cut into 1 mm cubes, dehydrated and infiltrated with LR-White resin, which was then polymerized at 50 °C for 24 h. Semi-thin sections were subsequently immunolabelled for AGT using rabbit anti-(human AGT) serum at a dilution of 1:25, and 10 nm diameter Protein A-colloidal gold particles, as previously described [1].

RESULTS

CpPEX5 encodes two isoforms like its human homologue

The open reading frames of the long and short *CpPEX5* cDNA clones pGPX5-7 and pGPX5-8 are 1920 and 1809 bp respectively. The nucleotide sequence of the long form has been deposited in the EMBL database under accession number AJ004953. They are predicted to encode polypeptides of 640 and 603 amino acids (Figure 2). The only difference in the polypeptides encoded by the clones is a 37-amino-acid insert in the longer isoform. Two putative splice-site variants have been found previously in *HsPex5p* [20,24,25] but not yeast *Pex5ps*, including those of *Hansenula polymorpha* [26,27], *Pichia pastoris* [28,29], *Yarrowia lipolytica* [30] and *S. cerevisiae* [10]. *CpPex5p* has an overall sequence identity of 97% and 94% with its *HsPex5p* and *MmPex5p* homologues, respectively, the only other mammals in which *PEX5* has been cloned [20,24,25,31]. However, *CpPex5p* is poorly conserved with respect to yeast *Pex5p*, being only 28% identical with *ScPex5p*. As with *HsPex5p*, eight TPR domains can be recognized in the C-terminal half of *CpPex5p* (Figure 2). The degree of conservation of this domain is only marginally better than that found with the whole protein (98%, 96% and 40% when compared with *HsPex5p*, *MmPex5p* and *ScPex5p* respectively). *CpPex5p* is predicted to be one amino acid larger than either *HsPex5p* or *MmPex5p*.

Transcripts for both the short and long forms of *Pex5p* are expressed in all guinea pig tissues

Little is known about the tissue-specific expression of *Pex5p* in mammals, especially the relative expression of the two splice variants. In order to determine whether mammalian *Pex5p* is

1	MAMRELVEGECGGANPLMKLAGHFTQDKALRQGLRPGWPPGAPASETVSKPLGVASED	Cp
1	T.....S.A.....GT..	Mm
1	A.....AA.....	Hs
61	ELVAEFLQDNAPLVSRAPQTTFKMDLLAEMQEIEQSNFRQAPQAPGVADLALSENWAQ	Cp
61	...S.....T.....	Mm
61	Q.....	Hs
121	EPLAAGDAVDVTQDYNETDWSQEFIAEVTDPVSVSPARWAEYLEQSEKILWLGEPEGAA	Cp
121	A.....H.....G.....DQ...FS	Mm
121	S.....T.....	Hs
181	TTDRWYDEYHPEEDLQHTASDFVAKVDDPKLANSEFLKFEVROIGEGQVSLSEVAGSGRAQ	Cp
181	S.....N.....A.....G.....	Mm
181	G.....Q.....	Hs
241	AEQWAAEFTIOOQGTSDAWVDQFTRPVN-TSALDMEFERAKSAIESDVFWDKLQAELEEM	Cp
241	E.....G.KIA...QV.....	Mm
240	QV.....	Hs
300	AKRDAEHPWLSDDYDLTSASYDKGYQFEENPLRDHPQPFEEGLRLLEEGDLPNAVLLF	Cp
301	H.....	Mm
299	T.....R.Q.....	Hs
360	BAAVQDQPKHMEAWQYLGTTQAEQBELLATISALRCLLEKPNRTALMALAVSFTNESL	Cp
361	R.....N.....S.....	Mm
359	Q.....	Hs
420	QRQACETLRDLRCTPAYAHLVTPAEAGAGGAGLSSKRIIGSLSDSLFLEVKEFLAA	Cp
421	YS.....A.G.....T.....P.....D.....	Mm
419	Y.....P.....	Hs
480	VRLDPTSIDPDVQCGLGVLFNLGSEYDKAVDCFTAALSVRPNDYLLWNKLGATLANGNQS	Cp
479	M.....	Mm
479		Hs
540	EEAVALYRALELQPGYIRSRYNLIGISCTNLGAHREAVHFLEALNMQRKSRGPRGEGGA	Cp
539		Mm
539		Hs
600	MSENIWTLRLALSMQLGQSDAYRAADARDLSALLALEGLSQ	Cp
599	G.....M.....P.....	Mm
599	G.....T.....TM...P.....	Hs

Figure 2 Comparison of the predicted *CpPex5p* amino acid sequence with those of other species

The deduced amino acid sequence of *CpPex5p* is compared with that of *MmPex5p* and *HsPex5p*. Residues identical with the guinea pig sequence are indicated by dots. Gaps in the sequence are shown by dashes. The eight TPR domains are underlined and numbered at the first residue of each. The 37-amino-acid insert present in mammalian *Pex5p*, but absent from yeast *Pex5p*, is shown double underlined.

expressed in all or only some tissues, and whether either variant is expressed preferentially in any tissues, we attempted to amplify by qualitative RT-PCR a region including the 111 bp insert in a wide variety of guinea pig tissues. Using the primers P11 and P12 (see Table 1), two products of 525 and 636 bp were amplified in all tissues studied, compatible with the presence of both long and short variants (Figure 3). Expression was greatest in the kidney, liver, adrenal gland, small intestine, heart and lung, and least in muscle, ovary and adipose tissue. The relative proportions of the short to long PCR products varied considerably, depending on the tissue used; whether this has any functional significance is unclear. Conservation of the putative splice-site variants in mammals suggests the requirement for both long and short forms. However, the only functional difference between them so far identified is that the long isoform is required for the peroxisomal import of PTS2 proteins, such as thiolase [32,33]. The reason for the conservation of the short isoform in mammals is unclear.

CpPEX5 cDNA can complement a human Δ *pex5* cell line

Both the long and short isoforms of *CpPEX5* cDNA (pGPX5-7 and pGPX5-8) encode functional PTS1 import receptors, as

Figure 3 Tissue-specific expression of the long and short isoforms of PEX5 mRNA

Total RNA was extracted from various guinea pig tissues. cDNA was prepared using the RT primer P10 (see Table 1) and then amplified using PCR primers P11 and P12. The products were then run on agarose gels and stained with ethidium bromide. In each case the top band is 636 bp and the bottom band is 525 bp; the difference in size being due to the presence or absence of the 111 bp insert. Guinea pig tissues analysed were: 1, liver; 2, kidney; 3, adrenal gland; 4, brain; 5, adipose tissue; 6, small intestine; 7, heart; 8, lung; 9, muscle; 10, ovary; 11, skin; 12, spinal cord; and 13, spleen. RNA (10 μ l) was used for RT-PCR, except for 1A and 2A where 1 μ l was used, and 1B and 2B where 5 μ l was used.

Figure 4 Complementations of catalase import into peroxisomes following microinjection of long and short isoforms of *CpPEX5* cDNA

Δ *pex5* cells were microinjected with either GFP cDNA alone (A and B) or GFP cDNA together with the long isoform of *CpPEX5* (C and D). After 24 h the distribution of endogenous catalase (B and D) was determined by immunofluorescence microscopy. The distribution of GFP (A and C) was determined by using its autofluorescence. Catalase is diffuse in uncomplemented cells (B), but punctate in those cells microinjected with long *CpPEX5* cDNA (D). GFP, lacking any targeting sequence, is diffuse (cytosolic) in all microinjected cells. In some cells, GFP was also found in the nucleus for reasons that are not immediately clear. The results obtained with short *CpPEX5* cDNA (results not shown) were identical with those obtained with long *CpPEX5* cDNA.

demonstrated by the fact that they can both complement peroxisomal import of endogenous catalase in a human Δ *pex5* fibroblast cell line (Figure 4), although the efficiency of complementation is highly variable and concentration dependent (see

Figure 5 Effect of Pex5p concentration on the distribution of AGT and GFP constructs and endogenous catalase in Δ *pex5* mutant human fibroblasts

PEX5 cDNA was injected into the nuclei of Δ *pex5* human fibroblasts at concentrations of 10 ng/ml, 500 ng/ml or 50 μ g/ml, together with cDNAs encoding AGT-KKL (A and B), AGT-SKL (C and D) or GFP-SKL (E and F), all at a concentration of 200 μ g/ml. Following culture for 24 h at 37 $^{\circ}$ C, the distributions of catalase (B, D and F) and AGT (A and C) were determined by immunofluorescence, whereas the distribution of GFP (E) was determined using its autofluorescence. Between 25 and 109 cells were counted for each group (i.e. long and short PEX5 constructs combined) at each concentration and the percentage of cells containing punctate rather than diffuse labelling was determined. To avoid counting cells that had not been microinjected, only cells expressing a reporter construct (i.e. GFP or AGT) were included in the analysis. Arrows indicate the typical percentage of normal cells with punctate labelling after injection of each reporter construct. Continuous lines correspond to *CpPEX5* cDNA (combined data for the short and long isoforms); broken lines correspond to *HsPEX5* cDNA (combined data for the short and long isoforms). The total cells counted were as follows: 10 ng/ml *CpPEX5*, 29 (AGT-KKL), 60 (AGT-SKL) and 26 (GFP-SKL); 500 ng/ml *CpPEX5*, 43 (AGT-KKL), 81 (AGT-SKL) and 57 (GFP-SKL); 50 000 ng/ml *CpPEX5*, 73 (AGT-KKL), 37 (AGT-SKL) and 25 (GFP-SKL); 10 ng/ml *HsPEX5*, 55 (AGT-KKL), 55 (AGT-SKL) and 37 (GFP-SKL); 50 ng/ml *HsPEX5*, 74 (AGT-KKL), 109 (AGT-SKL) and 59 (GFP-SKL); 50 000 ng/ml *HsPEX5*, 32 (AGT-KKL), 25 (AGT-SKL) and 28 (GFP-SKL).

below). In addition, both constructs can complement the peroxisomal import of AGT-KKL, AGT-SKL or GFP-SKL in such cells (see below).

CpPex5p is less efficient than *HsPex5p* at directing the peroxisomal import of proteins possessing non-consensus PTS1s, but not those possessing consensus PTS1s

The efficiency of import of endogenous catalase and transfected AGT-KKL, AGT-SKL and GFP-SKL depended on the expression level of Pex5p. When expression plasmids (encoding *CpPex5p* or *HsPex5p*; long or short) were injected at a DNA concentration of only 10 ng/ml, the percentage of cells that contained punctate catalase or AGT-KKL never rose above 10–15%. However, this rose to 70–90% when *HsPEX5* cDNA was injected at 500 ng/ml, but to only 25–35% when *CpPEX5* cDNA was injected at the same concentration (Figures 5A and 5B). No obvious differences were noticeable between the long and short isoforms. There were no clear differences between *CpPEX5* and *HsPEX5* cDNA at any concentration with respect

Figure 6 Immunofluorescence analysis of the competition between the peroxisomal import of GFP-SKL and catalase in complemented $\Delta pex5$ fibroblasts

$\Delta pex5$ cells were co-injected with GFP-SKL cDNA and cDNAs encoding either short *CpPex5p* (A–D) or long *CpPex5p* (E and F). After 24 h the distribution of GFP (A, C and E) was determined using its autofluorescence, and the distribution of endogenous catalase (B, D and F) was determined by immunofluorescence (see the Experimental section). Although GFP-SKL is punctate (i.e. peroxisomal) in almost all cells, catalase is mainly diffuse (B and F), even though some cells do show a punctate distribution pattern (D). A very similar picture was found with cDNAs encoding short and long *HsPex5p* (results not shown).

to the complementation of AGT-SKL or GFP-SKL peroxisomal import (Figures 5C and 5E).

Although the actual level of expression of Pex5p in these studies cannot be determined, the shapes of the dose–response curves and the enormous range of cDNA concentrations used suggest that the differences between the efficiency with which *HsPex5p* and *CpPex5p* can complement the peroxisomal import of catalase and AGT-KKL are unlikely to be due to different levels of expression.

Overexpression of *HsPex5p*, but not *CpPex5p*, inhibits the peroxisomal import of AGT-KKL, but not catalase or proteins possessing consensus PTS1s

The shape of the Pex5p dose–response curve over 3.5 orders of magnitude (10 ng/ml to 50 μ g/ml of plasmid DNA) was rather different depending on which construct was being studied. For all reporter constructs, as well as endogenous catalase, injecting PEX5 at a DNA concentration of only 10 ng/ml led to peroxisomal localization in only a small minority of the cells (see above; Figures 5A, 5B, 5C and 5E). However, when injected at a 50-fold greater concentration (i.e. 500 ng/ml) both long and short *HsPEX5* were able to complement the great majority of cells for all constructs and for endogenous catalase (in the absence of any exogenous competitor, see below). Under the conditions of the present experiments, 500 ng/ml would therefore be expected to saturate the peroxisomal import process (i.e. there is very little protein left to import), so that increasing the concentration further would have been expected to have had no, or at least a very minimal, effect. Indeed within the experimental

accuracy of the procedures employed, the import of GFP-SKL and endogenous catalase (in the absence of a competitor) appeared to have reached a plateau, so that little, or no, further increase in import was detectable after increasing the PEX5 cDNA concentration by an additional two orders of magnitude (i.e. 50 μ g/ml) (Figures 5B and 5E). Contrary to the situation with GFP-SKL and endogenous catalase, the peroxisomal import of AGT-KKL was markedly inhibited when either the long or short form of *HsPEX5*, but not *CpPEX5*, was injected at the highest concentration (50 μ g/ml) (Figure 5A). The dose–response curve for AGT-SKL appeared to be intermediate between that for AGT-KKL and GFP-SKL, with mild inhibition at high PEX5 cDNA concentrations (Figure 5C). Interestingly, even though *CpPEX5* only led to the complementation of AGT-KKL and catalase import in a minority of cells at a cDNA concentration of 500 ng/ml, no further import was seen even when the concentration was increased by two orders of magnitude to 50 μ g/ml (Figures 5A and 5B).

Peroxisomal import of endogenous catalase is inhibited by overexpression of proteins possessing the consensus PTS1 but not by AGT possessing a non-consensus PTS1

The PEX5 cDNA dose–response curve for the complementation of catalase peroxisomal import in $\Delta pex5$ cells was very similar irrespective of whether GFP (possessing no PTS1) or AGT-KKL (possessing a non-consensus PTS1) cDNA was co-injected (Figure 5B). However, if a reporter protein possessing the consensus PTS1 (i.e. AGT-SKL or GFP-SKL) was co-injected, then the ability of long or short *HsPEX5* or *CpPEX5* to

Figure 7 Interaction of AGT-KKL and AGT-SKL with *HsPex5p* in the yeast two-hybrid reaction, and their distribution in yeast cells

(A) The two-hybrid reaction was carried out in *S. cerevisiae* strain SFY526. cDNAs encoding AGT-KKL or AGT-SKL were fused to the GAL4 DNA-binding domain. These were used as partners to cDNA encoding the TPR domain of *HsPex5p* fused to the GAL4 transactivation domain (AD). Duplicate samples were run on an SDS/PAGE gel and blotted with antibodies against β -galactosidase. The control corresponds to the empty pGBT9 vector with the AD-Pex5p fusion. The only interaction discernible is that between AGT-SKL and Pex5p. (B) *S. cerevisiae* were transformed with AGT-KKL or AGT-SKL and grown on oleate. Cells were homogenized and fractionated into a high-speed pellet (p) and supernatant (s) fraction. AGT-KKL was found almost entirely in the supernatant fraction, whereas AGT-SKL was found more or less equally in both fractions.

complement the peroxisomal import of catalase was significantly compromised (Figures 5D and 5F). For example, following co-injection of long or short *HsPEX5* cDNA (500 ng/ml or 50 μ g/ml) and GFP or AGT-KKL cDNA, 80–100 % of the cells showed punctate catalase (Figure 5B). However, when co-injected at the same concentrations with GFP-SKL or AGT-SKL cDNA, only 10–40 % of cells showed any punctate catalase labelling (Figures 5D and 5F), despite most cells showing strong punctate labelling for the reporter (Figures 5C and 5E). A similar picture was also found with *CpPEX5* cDNA (Figure 6) at both 500 ng/ml and 50 μ g/ml, although in the latter case the overall level of complementation made the effect less obvious.

AGT-KKL does not interact, but AGT-SKL does, with *HsPex5p* in a yeast two-hybrid assay even though Pex5p is essential for its peroxisomal import

The results above, together with our previous observations [4,12], suggest that proteins containing non-consensus PTS1s, especially AGT-KKL, might interact rather differently with Pex5p than proteins containing consensus PTS1s (e.g. AGT-SKL and GFP-SKL). In order to test this possibility, we investigated the interaction between the TPR domain of *HsPex5p* and AGT-KKL or AGT-SKL using the yeast two-hybrid system. Our results (Figure 7A) showed that whereas AGT-SKL interacts with *HsPex5p* efficiently, AGT-KKL did not interact at all. This might indicate that the interaction between Pex5p and AGT-KKL is much weaker than that between Pex5p and AGT-SKL, or that the interaction involves parts of the Pex5p molecule other than the TPR domain. Alternatively, interaction of AGT-KKL and *HsPex5p* might involve additional factors not present in yeast cytosol. Interestingly, neither AGT-KKL nor AGT-SKL were able to interact with *ScPex5p* in this system (results not shown), even though many other peroxisomal proteins ending in SKL have been shown to do so.

Figure 8 Distribution of AGT-KKL and AGT-SKL in *S. cerevisiae*

S. cerevisiae were transformed with yAGT-KKL (A–C) or yAGT-SKL (D–F). Following 18 h of growth on medium containing oleate and Tween 40, but deficient in methionine, cells were fixed and labelled for AGT using the Protein A–gold technique [1]. Views of six different cells are shown in (A–F). Bars correspond to 0.1 μ m. The distribution of AGT-KKL (A–C) is cytosolic with no peroxisomal labelling detectable. In some cells, AGT-KKL was also found in the vacuole and intracellular aggregates (results not shown). AGT-SKL (D–F) is located in the matrix of organelles with characteristics typical of peroxisomes. The arrows in (D) indicate two peroxisomes.

KKL cannot direct the peroxisomal import of AGT in yeast, but SKL can

In an attempt to shed light on why AGT-KKL did not interact with *HsPex5p*, let alone *ScPex5p*, in the yeast two-hybrid system, we investigated whether it could be targeted to and imported into peroxisomes in *S. cerevisiae*. The results clearly showed that whereas the consensus PTS1 SKL could direct human AGT to peroxisomes in yeast, KKL could not (Figure 7B and Figure 8). In fact AGT-KKL remained mainly cytosolic or was localized to

vacuoles. The failure to target AGT-KKL to peroxisomes was unlikely to be the result of improper folding, as both AGT-SKL in the peroxisomes and AGT-KKL in the cytosol and vacuoles was catalytically active (results not shown).

DISCUSSION

Pex5p determines the efficiency with which AGT is imported into peroxisomes

Our previous studies had suggested that the apparent inefficiency with which AGT is imported into the peroxisomes of guinea pig cells might be due to a combination of the guinea pig peroxisomal import machinery and non-consensus PTS1s [4]. The results obtained in the present study confirm this suggestion and further show that the 'inefficient' component of the import system is likely to be its first component (i.e. Pex5p). Whether this is simply due to a lower affinity of *Cp*Pex5p, compared with *Hs*Pex5p, for non-consensus PTS1s, is currently not known, because even *Hs*Pex5p (at least the TPR domain of *Hs*Pex5p) did not interact with AGT-KKL in the yeast two-hybrid system, although it did interact with AGT-SKL. It is conceivable that other components of the peroxisomal import system (possibly currently unidentified) could also be involved in the guinea pig 'inefficiency response' (see below).

The use of the term 'inefficient' in a targeting context might be considered to be rather pejorative, but targeting inefficiency could be an advantage for the cell and have evolved as a way of localizing an enzyme to more than one compartment, particularly when one of the compartments is the cytosol. Many mechanisms have evolved in order to localize the same or similar metabolic reactions or other cellular processes to more than one part of the cell, including gene duplication, alternative transcription and translation initiation, and alternative splicing [34]. Inefficient, or incomplete, targeting could be added to the list.

Bona fide peroxisomal enzymes are frequently found in the cytosol where they can be catalytically active, the best example being catalase [35]. In some mammalian cells, which unlike yeast have only one catalase gene, > 90% of the enzyme might be cytosolic, even though all of the newly synthesized polypeptides are presumed to contain functional PTS1s. A function for cytosolic catalase as an H_2O_2 scavenger can easily be appreciated [36], but a specific role for cytosolic AGT is less obvious. We have previously suggested dual metabolic roles for AGT, namely glyoxylate detoxification in the peroxisomes and gluconeogenesis in the mitochondria [3]. Many components of the latter group of metabolic reactions can also be found in the cytosol. Whether guinea pigs need cytosolic AGT in addition to peroxisomal AGT, and why this species should have this need while many mammals do not, remain matters for speculation.

The observed import efficiency of an organellar protein is likely to be dependent upon many interdependent factors, including the affinity with which the protein ligand interacts with components of the import machinery, especially the receptor, the physical ability of the protein to transit the organelle membrane, the presence or absence of competing pathways, and the relative rates of degradation of imported and non-imported proteins. Although the extent to which these various factors might explain the presence or absence of peroxisomal proteins in the cytosol is in most cases unclear, it is recognized that some PTS1s are probably 'better' than others, at least in certain circumstances. For example, the dual peroxisomal/cytosolic distribution of epoxide hydrolase in rat liver has been attributed to the presence of a non-consensus PTS1 (i.e. Ser-Lys-Ile) [37]. In addition, on the basis of the differential effects of mutations in *Hs*Pex5p in peroxisomal biogenesis disease patients, Shimozawa et al. [38]

Table 2 Summary of the differences between AGT-KKL and AGT-SKL

Property	AGT-KKL	AGT-SKL
Efficiency of peroxisomal import in		
Human cells	++	+++
Guinea pig cells	+	+++
<i>S. cerevisiae</i>	—	+++
Interaction with <i>Hs</i> Pex5p in the yeast two-hybrid system?	No	Yes
Effect of high <i>Hs</i> Pex5p concentration on import	Strongly inhibited	Weakly inhibited
Effect on endogenous catalase import	No effect	Competes

suggested that some PTS1s were 'poorer' than others. For example, the Ala-Lys-Leu of bifunctional protein and sterol carrier protein 2 and the (Lys)-Ala-Asn-Leu of catalase were considered not to be as good as the Ser-Lys-Leu of acyl-CoA oxidase.

Despite the surprising variety of tripeptides found at the C-termini of matrix proteins imported via the PTS1 pathway (i.e. via Pex5p rather than Pex7p) and the frequency with which peroxisomal proteins are found in significant amounts in the cytosol, very little attention has been directed towards the molecular basis of variable peroxisomal import efficiency, a notable exception being the studies of Swinkels et al. [9]. It is generally found that non-*bona-fide* peroxisomal proteins (e.g. reporter proteins) and peroxisomal proteins expressed in non-natural environments (e.g. cells from different species) are less tolerant of variations from the archetypal PTS1 consensus motif of Ser-Lys-Leu etc. [39]. In some cases, the adjacent residues modify the acceptability of the C-terminal tripeptide [21], in others the species-specific Pex5p–PTS1 interactions might determine specificity and efficiency [40].

AGT is imported into peroxisomes differently from other PTS1 peroxisomal proteins

Compared with other peroxisomal proteins, AGT is fairly unusual. Firstly, although dependent on Pex5p (but not Pex7p) for its import into peroxisomes [12], its C-terminal tripeptides in both the human and guinea pig enzyme are not found in any other mammalian peroxisomal proteins. In fact, various screens in diverse systems have failed to identify either of these tripeptides as potential PTS1s [40,41]. In all mammals in which the AGT gene has been sequenced, the C-termini always have a two-out-of-three match with the various consensus possibilities [8,9]. For example, the sequence is Lys-Lys-Leu in the human and marmoset [13,15], Ser-Gln-Leu in the rabbit [15], Asn-Lys-Leu in the cat and rat [16,42], and His-Arg-Leu in the guinea pig [4]. We have shown previously that the C-terminal tripeptide of human AGT is necessary for peroxisomal import, but it is insufficient to direct the peroxisomal import of reporter proteins, such as GFP, bacterial chloramphenicol acetyltransferase and firefly luciferase [12,14]. This specificity is unusual and suggests that additional targeting information is required elsewhere in the AGT molecule. Unlike catalase, in which the lysine immediately adjacent to the C-terminal tripeptide provides all the necessary additional targeting information [21], in AGT this putative extra information is not contained within the adjacent 44 amino acids [14].

There are several pieces of evidence which suggest that the natural C-terminal tripeptides of AGT are not as good as the consensus PTS1 at directing the peroxisomal import of AGT (see Table 2), let alone the import of reporter proteins. Indeed,

the only direct evidence that these tripeptides are PTS1s at all is that peroxisomal import of AGT is dependent on the presence of Pex5p [12]. From this and previous data it would not be surprising to find that the functional interaction between AGT and its cognate receptor was different from that expected for other peroxisomal proteins. Two results from the present study also point in this direction. First, AGT-KKL did not compete with the import of endogenous catalase, whereas AGT-SKL and GFP-SKL did. Second, high concentrations of *HsPex5p* (both long and short) markedly inhibited the import of AGT-KKL, but much less so AGT-SKL, and did not inhibit the import of GFP-SKL or catalase at all. Although the former, together with the lower efficiency with which Lys-Lys-Leu, compared with Ser-Lys-Leu, can direct the peroxisomal import of AGT, could be explained by a weaker interaction with Pex5p (undetectable at the level of the yeast two-hybrid reaction), it would not explain the inhibitory effects of high *HsPex5p* concentrations.

An alternative explanation could be that the interaction of AGT-KKL with Pex5p requires an additional (adaptor) molecule. Depending on the kinetics, relative affinities of the components and stoichiometry of such a putative interaction, it is conceivable that high Pex5p concentrations could indeed compete with AGT for the adaptor. If Ser-Lys-Leu did not require the adaptor, or if it interacted with the adaptor with greater affinity than Lys-Lys-Leu, then most peroxisomal proteins would be less susceptible, or completely unaffected, by high concentrations of Pex5p. This putative adaptor might or might not be exclusive to AGT. If it was, then the failure of AGT-KKL to interact with *HsPex5p* in the *S. cerevisiae* two-hybrid system and its failure to be imported into the peroxisomes of *S. cerevisiae* could be the result of the adaptor being absent from yeast cells. The idea that a cytosolic factor, additional to the import receptor, might be required for peroxisomal targeting is not without precedent. For example, the peroxisomal import of the PTS2 protein thiolase in *Yarrowia lipolytica* requires a cytosolic factor (Pex20p) in addition to the import receptor Pex7p [43].

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