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PTS1-independent targeting of isocitrate lyase to peroxisomes requires the PTS1 receptor Pex5p

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Summary

The targeting of castor bean isocitrate lyase to peroxisomes was studied by expression in the heterologous host Saccharomyces cerevisae from which the endogenous ICL1 gene had been removed by gene disruption. Peroxisomal import of ICL was dependent upon the PTS1 receptor Pex5p and was lost by deletion of the last three amino acids, Ala-Arg-Met. However, removal of an additional 16 amino acids restored the ability of this truncated ICL to be targeted to peroxisomes and this import activity, like that of the full-length protein, was dependent upon Pex5p. The ability of peptides corresponding to the carboxyl terminal ends of wild-type and $\Delta 3$ and $\Delta 19$ mutants of ICL to interact with the PTS1-binding portion of Pex5p from humans, plants and yeast was determined using the yeast twohybrid system. The peptide corresponding to wild-type ICL interacted with all three Pex5p proteins to differing extents, but neither mutant could interact with Pex5p from any species. Thus, ICL can be targeted to peroxisomes in a Pex5p-dependent but PTS1-independent fashion. These results help to clarify the contradictory published data about the requirement of the PTS1 signal for ICL targeting.

Keywords: Peroxisome; targeting signal; glyoxylate cycle 'piggyback import'.

Introduction

Peroxisomes play vital metabolic roles in eukaryotic cells. Their biochemical repertoire is very diverse and includes common functions such as β -oxidation of fatty acids and detoxification of reactive oxygen species as well as specialized functions found only in some organisms. The latter include the glyoxylate cycle (plants and micro-organisms), photorespiration (plants), degradation of phytanic acid and bile salts (mammals), and ether phospholipid biosynthesis (mammals) (Van den Bosch *et al.* 1992). The importance of

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peroxisomes is emphasized by the consequences of mutations that disable peroxisome assembly and function. In humans such mutations are responsible for a group of rare but devastating diseases called the peroxisome biogenesis disorders (PBDs) (Wanders 1999). In plants, mutants in peroxisome function affect germination, seedling establishment and growth (Hayashi et al. 1998, Eastmond et al. 2000, Germain et al. 2001, Liepman and Olsen 2001). In singlecelled organisms, the consequences of peroxisome function are less severe, resulting instead in a failure to grow on carbon sources such as fatty acids that require peroxisomal metabolic pathways. Consequently, a number of mutants, designated pex mutants, have been isolated from Saccharomyces cerevisiae and other yeasts (Distel et al. 1996). The study of the corresponding genes and proteins and their use to identify orthologues in other organisms has provided a wealth of information about peroxisome protein targeting and organelle biogenesis (Sacksteder and Gould 2000, Subramani et al. 2000, Purdue and Lazarow 2001).

All peroxisomal proteins are encoded by nuclear genes and need to be delivered accurately to the peroxisome. Matrix proteins carry one of two targeting signals, PTS1, a carboxyl-terminal tripeptide, and PTS2, an amino-terminal nonapeptide. These signals are recognized by their cognate receptors, Pex5p and Pex7p, respectively, in the cytosol and delivered to the peroxisomes. Pex5p and Pex7p loaded with their respective cargoes dock at the peroxisome membrane at a complex consisting of Pex13p and Pex14p (and Pex17p in S. cerevisiae). Following docking the cargo is translocated across the peroxisome membrane in a still ill-defined reaction requiring ATP hydrolysis and a number of other proteins including Pex2p, Pex10p and Pex12p (reviewed in Sparkes and Baker 2002). A small number of proteins, for example S. cerevisiae Acyl CoA oxidase, appear to possess neither a PTS1 nor PTS2 signal, raising the possibility that an additional signal, so-called PTS3, is involved in the targeting of these proteins. Other proteins although possessing a functional PTS1 signal are still capable of being transported to peroxisomes after the PTS1 signal has been deleted (Kragler et al. 1993, Elgersma et al. 1995). One characteristic of peroxisomes is their ability to import folded, even oligomeric, proteins and it has been established that some proteins that lack an appropriate targeting signal can be delivered to peroxisomes by association with a subunit that posseses a functional targeting signal. This has been referred to as 'piggy back' import (Glover et al. 1994, McNew and Goodman 1994).

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The glyoxylate cycle is a peroxisomal metabolic pathway required for the use of two-carbon compounds for growth (Kornberg and Krebs 1957). The two unique steps of the glyoxylate cycle catalayed by isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) bypass the oxidative decarboxylation steps catalysed by isocitrate dehydrogenase and α -ketoglutarate dehydrogenase of the Krebs cycle. Mammals lack these enzymes and as a consequence cannot carry out gluconeogenesis from fatty acids. In plants and micro-organisms that posses this pathway, acetyl CoA from fatty acid β -oxidation (and other two carbon compounds such as acetate and ethanol) can be converted to malate and used for gluconeogenesis (Beevers 1961).

The targeting of isocitrate lyase from plants has been studied by several laboratories, but different results were obtained. Olsen and Harada showed that removal of the last 37 amino acids of oilseed rape ICL abolished targeting to peroxisomes in transgenic Arabidopsis plants (Olsen *et al.* 1993). In contrast, removal of the last 19 amino acids of castor bean ICL did not prevent targeting to peroxisomes in an *in vitro* system (Behari and Baker 1993), in transgenic tobacco plants (Gao *et al.* 1996) or when expressed heterologously in *S. cerevisiae* (Taylor *et al.* 1996). Removal of the last three amino acids from castor bean and cotton ICL resulted in a failure to target the protein to peroxisomes in tobacco cell cultures, but co-expression of native subunits with those lacking the last three amino acids could restore import of this mutant protein (Lee *et al.* 1997).

In order to understand the different behaviour of the various ICL mutants, we expressed full-length ICL and two carboxyl terminal deletions that remove the last three and last 19 amino acids in *S. cerevisiae* mutants that lack the PTS1 receptor and PTS2 receptor, respectively. To exclude 'piggy backing', all strains contained a disrupted *S. cerevisiae* ICL1 gene. Our results show that while removal of the last three amino acids of ICL abolishes targeting, the ICL Δ 19 protein regains its targeting ability and that this is dependent on the PTS1 receptor Pex5p. Further, the carboxyl terminus of this mutant protein does not interact with the PTS1-binding part of Pex5p in a yeast two-hybrid assay.

Results

Verification of the ICL knockout strains

Isocitrate lyase is a tetrameric enzyme. Lee *et al.* (1997) presented evidence that castor bean ICL lacking its PTS1 sequence ARM could be piggy-backed into peroxisomes of tobacco BY2 suspension cells by co-expression with a wild-type subunit. *S. cerevisiae* ICL is a cytosolic enzyme (Taylor *et al.* 1996), but in order rigorously to exclude the possibility that some small proportion of the yeast enzyme is imported into peroxisomes and can carry the castor bean subunit with it, and to eliminate immunological cross-reactivity from the endogenous ICL, all the experiments were carried out in an ICL null background. Isocitrate lyase is an essential enzyme for the functioning of the glyoxylate cycle that permits *S. cerevisiae* to grow on two-carbon compounds such as acetate and ethanol. Figure 1 shows that the parent strain



Potassium acetate Media



Figure 1. Phenotypes of the isocitrate lyase deletion strains. In each of the top three panels, (a) is BSL1-11B; (b) is BSL1-11B icl1 Δ ; (c) is BSL1-11B pex7 Δ icl1 Δ ; and (d) is BSL1-11B pex5 Δ icl1 Δ . The top panel shows that all four strains grow on glucose-containing media (YPD). The second and third panels shows that once the endogenous S. cerevisiae ICL1 gene has been knocked out, the strains can no longer grow on potassium acetate or ethanol media as these carbon sources require a functional glyoxylate cycle. Plates were incubated for 24 h where growth was observed or for 7 days for plates where no growth was observed. (bottom) Total protein was extracted from BSL1-11B icl1 Δ (lane 1) BSL1-11B pex7 Δ icl1 Δ (lane 2) BSL1-11B pex5 Δ icl1 Δ (lane 3) and BSL1-11B (lane 4) cells that had been precultured on minimal glucose and subsequently transferred to oleic acid medium overnight. Protein extracts were separated by SDS-PAGE, transferred to nitrocellulose and detected with anti-castor bean ICL antibody.

BSL1-11B can grow on both potassium acetate and ethanol as the sole carbon source. In contrast, BSL1-11B icl1 Δ , BSL1-11B pex5 Δ icl1 Δ and BSL1-11B pex7 Δ icl1 Δ could grow on glucose-containing YPD but not on potassium acetate or ethanol. Total protein was extracted from each of the strains and assayed for ICL activity and protein. In the control strain ICL activity was 466 µmol min⁻¹ mg⁻¹, whereas the ICL null mutants had undetectable activity. Consistent with this, Icl1p was readily detectable in the control but absent from the mutants by immunoblotting (Figure 1 bottom).

Proper targeting of castor bean ICL requires the PTS1 receptor but not the PTS2 receptor

Plasmid pEMBLYEX4 ICL encoding full-length castor bean isocitrate lyase under the control of a galactose inducible promoter was introduced into BSL1-11B pex5 Δ icl1 Δ and BSL1-11B pex7 Δ icl1 Δ . The resulting transformants were selected on uracil-free media and checked for galactose-inducible expression of Icl1p (data not shown). Single colonies were picked from selective medium and precultured in miminal glucose medium for 24–30 h before transfer into media containing 0.1% v/v oleic acid and 0.05% w/v galactose for a further 18–24 h. Although neither pex5 or pex7 mutant can grow on oleate, oleate triggers the synthesis of many peroxisomal enzymes and proteins. A total of 0.05% galactose is sufficient to induce ICL expression but it

does not cause significant repression of peroxisomes (Taylor *et al.* 1996). A post-nuclear supernatant was prepared from cells grown in this manner and separated on a 32–60% (w/w) stepped sucrose gradient. The localization of peroxisomes and mitochondria in the gradient was determined by the measurement of catalase, a peroxisomal protein, and fumarase, a mitochondrial matrix marker, as well as by immunoblotting for 3-ketoacyl thiolase, a PTS2-targeted peroxisome matrix protein. Castor bean ICL is not enzymatically active when expressed in *S. cerevisiae* (Taylor *et al.* 1996) so it was detected by immunoblotting.

In BSL1-11B pex7 Δ icl1 Δ cells (Figure 2) catalase (black squares) is found predominantly toward the bottom of the gradient with a peak in fraction 31 (48% w/w sucrose). This is very similar to the density of peroxisomes in cells with a functional PTS2 receptor (cf. Figure 4). This is because thiolase is the only known PTS2-targeted protein in *S. cerevisiae*, therefore its mislocalization does not affect peroxisome density significantly. A smaller peak of catalase



Figure 2. Isocitrate lyase is sorted to peroxisomes in the absence of the PTS 2 receptor and is protease protected. BSL1-11B pex7 Δ icl1 Δ cells transformed with a plasmid containing castor bean ICL under the control of a galactose-inducible promoter were grown on minimal glucose then transferred to 0.1% oleic acid 0.05% galactose media as described in the Experimental procedures. A post-nuclear supernatant was prepared and separated by centrifugation through a sucrose gradient. (a) The gradient was fractionated and the fractions assayed for catalase (peroxisome marker; closed squares), fumarase (mitochondrial marker; closed triangles) and total protein (crosses). Sucrose concentration of the fractions is also shown (solid circles). (b, c) Equal volumes of fractions corresponding to those in (a) were separated by SDS-PAGE and immunoblotted with antibodies raised against castor bean ICL and *S. cerevisiae* thiolase, respectively. (d) Fractions corresponding to the peroxisomes from a similar gradient to that shown in (a) were pooled and divided into three equal portions. Lanes 1, no treatment; 2, treated with 0.01 mg ml⁻¹ thermolysin in the presence of 0.5% v/v Triton-X 100.

activity was detected at the top of the gradient and it represents enzyme released from peroxisomes damaged during cell breakage. Mitochondria are well resolved from peroxisomes as the mitochondrial marker fumarase (black triangles) peaks in fraction 19 (42.5% w/w sucrose). As expected, thiolase is only detected in the cytosolic fractions at the top of the gradient (Figure 2c) confirming that this is a pex7 mutant. In contrast, castor bean ICL is efficiently targeted to the peroxisomal fraction (Figure 2b), its distribution co-inciding perfectly with that of catalase. In order to establish that ICL is actually imported into and not just associated with peroxisomes, a sample from the peak peroxisomal fraction of a gradient similar to that shown in Figure 2a was subjected to protease treatment. Treatment of intact peroxisomes with thermolysin (Figure 2d, lane 2) resulted in almost complete protection of isocitrate lyase, whereas in the presence of thermolysin and Triton-X 100 it was completely degraded (Figure 2d, lane 3). An identical result was obtained when ICL was expressed in BSL1-11B icl1 Δ (data not shown). Therefore, ICL does not require the PTS2 receptor for proper targeting.

When the experiment was repeated in BSL11-11B pex5 Δ icl1 Δ cells a very different result was obtained (Figure 3). In pex5 Δ mutants all PTS1 proteins are mislocalized to the cytosol but thiolase is correctly targeted. As the majority of proteins are PTS1 targeted, this results in peroxisomes of an abnormally low density as shown by thiolase which peaks in fraction 9 (Figure 3c, 37–39% w/w sucrose). As expected for

a Pex5p-dependent targeted protein, catalase was found exclusively at the top of the gradient, consistent with its mislocalization to the cytosol (Figure 3a). The mitochondrial marker fumarase was unchanged in its distribution (Figure 3a). Castor bean ICL, like catalase, is found exclusively at the top of the gradient (Figure 3b). Therefore, castor bean ICL is targeted by a Pex5p-dependent pathway in *S. cerevisiae*.

Removal of the c terminal tripeptide ARM-COOH abolishes peroxisomal targeting in S. cerevisiae

It has previously been reported that the carboxy-terminal tripeptide of castor bean ICL is a functional PTS in tobacco cells (Lee *et al.* 1997). ICL Δ 3, which lacks these three amino acids, was expressed in BSL1-11B icl1 Δ (Figure 4). Catalase (Figure 4a) and thiolase (Figure 4c) co-localized, with a peak in fraction 27 (48% w/w sucrose). In contrast, ICL Δ 3 was exclusively in the top three fractions, demonstrating that it was not targeted to peroxisomes.

ICL \varDelta 19 requires the PTS1 receptor for targeting to peroxisomes

Despite ICL Δ 19 lacking the C-terminal PTS1 sequence ARM-COOH, it can be imported into peroxisomes *in vitro* in transgenic tobacco plants (Gao *et al.* 1996) and in *S. cerevisiae* (Taylor *et al.* 1996). To begin to understand how



Figure 3. Isocitrate lyase is dependent on the PTS1 receptor for targeting to peroxisomes. BSL1-11B pex5 Δ icl1 Δ cells transformed with a plasmid containing castor bean ICL under the control of a galactose inducible promoter were grown on minimal glucose then transferred to 0.1% oleic acid 0.05% galactose media as described in the Experimental procedures. A post-nuclear supernatant was prepared and separated by centrifugation through a sucrose gradient. (a) The gradient was fractionated and the fractions assayed for catalase (peroxisome marker; closed squares), fumarase (mitochondrial marker; closed triangles) and total protein (crosses). Sucrose concentration of the fractions is also shown (solid circles). (b, c) Equal volumes of fractions corresponding to those in (a) were separated by SDS-PAGE and immunoblotted with antibodies raised against castor bean ICL and S. *cerevisiae* thiolase, respectively.



1 3 5 7 9 11 13 15 1719 21 23 25 27 29 31 33 35 37 39

Figure 4. Removal of the c terminal tripeptide ARM-COOH abolishes peroxisomal targeting. BSL1-11B icl1 Δ cells transformed with a plasmid in which castor bean ICL lacking the last three amino acids (-ARM) was under the control of a galactose inducible promoter, were grown on minimal glucose then transferred to 0.1% oleic acid 0.05% galactose media as described in the Experimental procedures. A post-nuclear supernatant was prepared and separated by centrifugation through a sucrose gradient. (a) The gradient was fractionated and the fractions assayed for catalase (peroxisome marker; closed squares), fumarase (mitochondrial marker; closed triangles) and total protein (crosses). Sucrose concentration of the fractions is also shown (solid circles). (b, c) Equal volumes of fractions corresponding to those in (a) were separated by SDS-PAGE and immunoblotted with antibodies raised against castor bean ICL and *S. cerevisiae* thiolase, respectively.

ICL Δ 19 is targeted when ICL Δ 3 is not, it was expressed in BSL1-11B icl1 Δ , BSL1-11B pex7 Δ icl1 Δ and BSL1-11B pex5 Δ icl1 Δ cells and the subcellular distribution was determined as before. In BSL1-11B pex7 Δ icl1 Δ cells (data not shown) as in BSL1-11B icl1 Δ cells (Figure 5a, b), ICL Δ 19 was efficiently targeted to peroxisomes. It co-localized perfectly with catalase at a density of 47% w/w sucrose (Figure 5a, b). In marked contrast, when expressed in BSL1-11B pex5 Δ icl1 Δ cells, ICL Δ 19 behaved identically to fulllength ICL (cf. Figure 3) and was not targeted to peroxisomes. It was found exclusively in the top fractions of the gradient (Figure 5d) and was clearly resolved from thiolase (Figure 5e), which is the peroxisome marker in this strain. Thus, although ICL∆19 lacks the carboxy terminal ARM-COOH, which is both necessary and (Figure 4) and sufficient (Lee et al. 1997) to function as a PTS1, it can be targeted efficiently to peroxisomes in a Pex5p-dependent manner.

Carboxyl terminus of ICL∆19 is not a PTS1

One possible explanation is that removing 19 amino acids from the carboxyl terminus of ICL fortuitously creates a new PTS1. PTS1 sequences can be quite divergent in both *S. cerevisiae* (Elgersma *et al.* 1996, Lametschwandtner *et al.* 1998) and plants (Mullen *et al.* 1997) although the carboxyl terminal sequence of ICL Δ 19 -MGKGVTEEQFKETWTR-COOH does not bear any resemblance to any reported PTS1 sequence. Pex5p binds PTS1 sequences via a series of tetratricopeptide repeats (TPRs) located in the carboxyl terminal half of the protein. The TPR repeats fold as a separate domain and are functionally interchangeable between species and responsible for PTS-1 recognition (Gatto *et al.* 2000, Gurvitz *et al.* 2001). The TPR-domains from *S.*

cerevisiae (pAH987), Nicotinia tabacum (pAH9875) and Homo sapiens (hP87) Pex5p fused to the GAL4-binding domain (Kragler et al. 1998, Lametschwandtner et al. 1998) were tested for interaction with peptides derived from the carboxyl-termini of the three castor bean ICL constructs fused to the GAL activation domain in the yeast two-hybrid system. As a control, a peptide originally identified as an interaction partner with the human TPR-domain was used. The pairwise interactions were quantified by the measurement of β-galactosidase activity. The results are presented in Table 1. All controls (pICL1, pICL2, pICL3, Hs01 with the empty binding domain vector and pAH9875, pAH987, hP87 with the empty activation domain vector) were below the detection limit (data not shown). ICL1 corresponding to the native carboxyl terminus of castor bean ICL interacted efficiently with both tobacco and human Pex5p, but only weakly with S. cerevisiae Pex5p. Neither of the peptides corresponding to the carboxyl terminus of ICL Δ 3 (ICL3), or ICL Δ 19 (ICL2) showed interaction with Pex5p from any of the species tested.

Discussion

Full-length castor bean isocitrate lyase requires the PTS1 receptor Pex5p for targeting to peroxisomes in *S. cerevisiae* (Figure 3). Deletion of the last three amino acids-ARM abolished targeting (Figure 4). However, removing a further 16 amino acids from the carboxyl terminus to create ICL Δ 19 resulted in a protein that was efficiently targeted *in vitro* (Behari and Baker 1993), *in planta* (Gao *et al.* 1996) and in *S. cerevisiae* (Taylor *et al.* 1996). In this paper, we demonstrate conclusively that this targeting cannot be by



Figure 5. Isocitrate lyase lacking the last 19 amino acids requires the PTS1 receptor for sorting to peroxisomes. BSL1-11B icl1 Δ cells transformed with a plasmid in which castor bean ICL lacking the last 19 amino acids was under the control of a galactose-inducible promoter were grown on minimal glucose then transferred to 0.1% oleic acid 0.05% galactose media as described in the Experimental procedures. A post-nuclear supernatant was prepared and separated by centrifugation through a sucrose gradient. (a) The gradient was fractionated and the fractions assayed for catalase (peroxisome marker; closed squares), fumarase (mitochondrial marker; closed triangles) and total protein (crosses). Sucrose concentration of the fractions is also shown (solid circles). (b) Equal volumes of fractions corresponding to those in (a) were separated by SDS-PAGE and immunoblotted with antibodies raised against castor bean ICL. (c) ICL Δ 19 was expressed in BSL1-11B pex5 Δ (peroxisome marker; closed squares), fumarase (mitochondrial marker; closed and the fractions assayed for catalase (peroxisome marker; closed for (a). The gradient was fractionated and the fractions assayed for catalase (peroxisome marker; closed squares), fumarase (mitochondrial marker; closed and the fractions assayed for catalase (peroxisome marker; closed squares), fumarase (mitochondrial marker; closed triangles) and total protein (crosses). Sucrose concentration of the fractions as described for (a). The gradient was fractionated and the fractions assayed for catalase (peroxisome marker; closed squares), (d) Equal protein and (e) equal volumes of fractions corresponding to those in (c) were separated by SDS-PAGE and immunoblotted with antibodies raised against castor bean ICL and *S. cerevisiae* thiolase, respectively.

virtue of association and co-import with endogenous lcl1p, as all the experiments have been carried out in strains in which the endogenous ICL has been disrupted. Furthermore, although ICL Δ 19 targeting has an absolute requirement for the PTS1 receptor (Figure 5), a peptide corresponding to its carboxyl terminal end is unable to interact with the TPR domain of Pex5p from tobacco, human or yeast in the yeast two-hybrid system (Table 1). From these results, it is concluded that the functional interaction of ICL Δ 19 with Pex5p is independent of its carboxyl terminus. In this respect ICL behaves similarly to *Saccharomyces* carnitine acetyl transferase (Cat2p). This protein can be targeted to peroxisomes by virtue of a C-terminal PTS1. However, when this PTS1 sequence is deleted, Cat2p is still targeted to peroxisomes and is still capable of interacting with Pex5p in the yeast two-hybrid assay (Elgersma *et al.* 1995). *S. cerevisiae* catalase A(CTA1p) is also capable of being imported without its C-terminal tripeptide (Kragler *et al.* 1993), but as shown in this paper (Figure 3a) targeting is also Pex5p-dependent.

Other proteins have been described that lack either PTS1 or PTS2 sequences. *S. cerevisiae* acyl CoA oxidase (Pox1p) was recently shown to interact directly with Pex5p, but in a

Table 1. Interactions between hexadecameric peptides representing a canonical PTS1 (Hs01), the C-terminus of full-length ICL (ICL1), the C-terminus of ICL Δ 19 (ICL2) or the C-terminus of ICL Δ 3 (ICL3) and TPR domains of Nt (pAH9875), Sc (pAH987) or Hs (hP87).

Peptide	Name	pAH9875	pAH987	hP87
-GPVEIGLDWLVVISKL	Hs01	2230+/-320	225+/-30	2450+/-345
-MEMGSAGSEVVAKARM	ICL1	880+/-63	16+/-2	1065+/-40
-MGKGVTEEQFKETWTR	ICL2	<5	<5	<5
-PGAMEMGSAGSEVVAK	ICL3	<5	<5	<5

Table 2. Strains used in this study.

Strain name	Genotype	Reference
BSL1-11B	MATa, lys2, his4-519, ura3 Δ , leu2-2, 112.	Taylor <i>et al</i> . (1996)
BSL1-11B icl1Δ	MATa, lys2, his4-519, ura3 Δ , leu2-2, 112, Δ ICL1::KanMX4	this study
BSL1-11B pex5Δ icl1Δ	MATa, lys2, his4-519, ura3 Δ , leu2-2, 112, Δ ICL1::KanMX4 Δ pex5::LEU2	this study
BSL1-11B pex7Δ icl1Δ	MATa, lys2, his4-519, ura3 Δ , leu2-2, 112, Δ ICL1::KanMX4 Δ pex7::LEU2	this study
BSL1-11B pex7Δ	MATa, lys2, his4-519, ura3 Δ , leu2-2, 112, Δ Pex7::LEU2	this study

manner distinct from conventional PTS1-targeted proteins. Amongst several lines of evidence supporting this conclusion, a mutant Pex5p (N393D) defective in targeting conventional PTS1 proteins could still interact with Pox1p in the yeast two-hybrid system and mediate its import whereas a mutant Y253N was specifically defective in Pox1p import (Klein *et al.* 2002). The region of Pox1p that binds Pex5p was not established, but it did not lie within the carboxyl terminal 17 amino acids.

An alternative means for a protein that lacks a functional PTS1 to enter peroxisomes is by co-import with a subunit containing a functional PTS1. Although association and co-import of castor bean ICL with endogenous yeast ICL can be excluded, a recent study has showed that *S. cerevisiae* $\Delta^2 \Delta^3$ -enoyl-CoA isomerase (Eci1p) could be co-imported with Dci1p, a peroxisomal PTS1 targeted protein that shares 50% identity with Eci1p (Yang *et al.* 2001).

What mechanism could be operating in the case of ICL? Targeting is lost in ICL Δ 3 but regained in ICL Δ 19 and this mechanism appears to be conserved in plants and yeasts as ICL Δ 19 is targeted when expressed in tobacco (Gao *et al*. 1996) but ICL Δ 3 is not (Lee *et al.* 1997). This suggests that removal of those additional 16 amino acids results either in a conformational change or in the removal of the binding site of some masking factor that allows interaction with Pex5p or another PTS1 targeted protein. However, a further deletion to amino acid 539 in the virtually identical Brassica napus ICL resulted in loss of targeting (Olsen et al. 1993). The twohybrid data (Table 1) show that the native C-terminus of ICL is a good ligand for human and tobacco Pex5p but a poor one for Saccharomyces Pex5p. ARM may therefore be a poor PTS-1 in yeast, raising the possibility that in yeast (but not plants) even the native protein may be targeted by the novel Pex5p interaction demonstrated for ICLA19. In this scenario, the folding/conformation of the ICLA3 polypeptide would be incompatible with this novel targeting mechanism.

The three-dimensional structure of *Aspergillus nidulans* ICL has recently been determined by X-ray diffraction (Britton *et al.* 2000). Unfortunately, the carboxy-terminal

most 18 residues (amino acids 521-538 in the Aspergillus sequence, which corresponds to 533 onwards in the castor bean sequence) cannot be traced in the structure suggesting that they are unstructured and mobile. However, the structure does show that there are extensive contacts between the carboxy-terminal helices and other parts of the opposite subunit in the tetramer (Table 1) (Britton et al. 2000). It is possible that deletions at the carboxyl terminus such as those made to study ICL targeting could interfere with tetramerization. Residues normally buried at the subunit interfaces could become exposed in the ICLA19 mutant, allowing interaction with either Pex5p in a non-conventional manner or with another PTS1 targeted protein. One candidate is malate synthase, which catalyses the subsequent step of the glyoxylate cycle and has been reported to interact with isocitrate lyase from maize (Beeckmans et al. 1994). MIs1p is targeted to peroxisomes in S. cerevisiae via the PTS1 pathway only when cells are grown on oleic acid (Kunze et al. 2002). However, import via MLS1p seems unlikely given that S. cerevisiae ICL is cytosolic in cells grown on oleate (Taylor et al. 1996). An alternative possibility is that mutations that destabilize the quaternary structure may allow more efficient import by a normally weak secondary targeting signal. Monomeric ICL has been reported to be imported in vitro more efficiently than oligomeric ICL (Crookes and Olsen 1998). Whatever the mechanism, loss of targeting in the construct studied by (Olsen et al. 1993) might be explained if this further deletion affected the folding or stability of the monomer and therefore its interaction with whatever protein brings about targeting. To resolve this issue will require an analysis of the oligomerization status of ICL and its various mutants and identification of proteins that interact with them.

Experimental procedures

Construction of yeast strains

The genotypes of the strains used are shown in Table 2. BSL1-11B pex7 Δ was constructed by transformation of BSL1-11B with J. A. Parkes et al.

linearized plasmid pJJGD7 (Marzioch et al. 1994) in which nucleotides 160-1275 of the PEX7 ORF were replaced with LEU2. Colonies were selected on leucine-free media and checked for failure to grow on oleic acid media. The disruption was confirmed by PCR on genomic DNA extracted from mutant and wild-type, using gene-specific primers PEX7S1 and PEX7AS2 (5'-TTTGAATTCGAACGCTCGG-3' and 5'-TAAT-TACGTAAATGATCTGCC-3', respectively). These primers flank the site of the LEU2 insertion. The expected fragments of 2.19 and 1.30 kb were observed in mutant and parental strain, respectively. BSL1-11B icl1 Δ and BSL1-11B pex7 Δ icl1 Δ were constructed using the KanMX4 cassette. Primer ICLKO S (5'-ATGCCTATCCCCGTTGGAAATACGAAGAACGATTTTG-CAGCTTTACGTACGCTGCAGGTCGAC-3') contains 18 bases at the 3' end homologous to the multiple cloning site flanking the KanMX4 gene in plasmid pFA6a (Wach et al. 1994) and 45 bases homologous to the coding strand of *S. cerevisiáe* ICL at the 5' end of the ICL ORF. ICLKO AS (5'-TTTCTTTACGC-CATTTTCTTTGAATTGATCTTCTGTGACACCCGTCGAGCTC-GAATTCATCGAT-3') contains 19 bases homologous to the multiple cloning site on the opposite side of the KanMX4 gene and 45 bp homologous to the non-coding strand at the 3' end of the S. cerevisiae ICL ORF. The PCR product was transformed into BSL1-11B pex7 Δ and BSL1-11B cells by electroporation (Becker and Guarente 1990) and mutants selected for further characterization by their ability to grow on YPD (2% glucose, 2% yeast extract, 1% peptone) in the presence of geneticin G418 (200 mg I⁻¹). BSL1-11B pex5 Δ icl1 Δ was constructed by knocking out the PEX5 gene in the BSL1-11B icl1∆ strain as follows. The LEU2 gene was amplified by PCR from plasmid YEp13 using primers PEX5 S1 (5'-CCATCGATGATAAAAGAA-GAATGGAAATAGGCCATCCTCAGGCAGGCCTCGAGGAGA-3') and PEX5 AS2 (5'-CCGAGCTCTAACAAAAGAAGG-TTTTAGTTGTAGTGCCCTATGATAGGCGTCGACTACGTCG-TAAGGCCG-3'). These primers contain 18 and 23 bases homologous to the 5' and 3' ends of the LEU2 ORF, respectively, and 46 bp homologous to the 5' and 3' ends of the $\dot{\text{PEX5}}$ ORF. BSL1-11B icl1∆ was transformed with the PCR product by electroporation and transformants selected on leucine-free media.

Plasmids

pEMBLYEX4-ICL and pEMBLYEX4ICL Δ 19 have been described previously (Taylor *et al.* 1996) and encode full-length or truncated ICL under the control of a galactose-inducible promoter. To construct pEMBL YEX4-ICL Δ 3 primer ICL Δ 3AS (5'-CAGT<u>CTGCAGTTACTTGGCAACCACCTCGCTCC-3'</u>) was designed to mutate the Ala residue at position 574 in the castor bean ICL protein to a stop codon, deleting the last three residues, Ala-Arg-Met. The ICL cDNA was amplified from plasmid pAB102 (Behari and Baker 1993) using ICL Δ 3AS and ICL Δ 3 S (5'-C<u>GAGCTCGGTACCCAGC-3'</u>) and *Taq/Pwo* polymerase. The product was cloned into pGEM-T-easy and sequenced, then excised using the *Sst1* and *Pst1* sites introduced by the primers (underlined), and ligated with *Sst1-Pst1*-digested pEMBLYEX4.

Media, cell growth and subcellular fractionation

Cells were grown on minimal glucose medium (WOYD; 0.67% (w/v) yeast nitrogen base with amino acids, 0.1% (w/v) yeast extract, 0.3% (w/v) glucose), transferred to oleate medium containing 0.05% (w/v) glactose (WOYOGal; 0.67% (w/v) yeast nitrogen base with amino acids, 0.1% (w/v) yeast extract, 0.05% (v/v) Tween 20, 0.05% (v/v) oleic acid, 0.05% (w/v) glactose), converted to spheroplasts and broken as described in Taylor *et al.* (1996). A post-nuclear supernatant (1 ml) was applied to a 38-ml stepped w/w sucrose gradient (5 ml 32%, 5 ml 35%, 9 ml 40%, 10 ml 45%, 5 ml 50%, 4 ml 60%). Sucrose solutions were made up in 10 mM MES-KOH, pH 6.0, 10 mM KCl, 1 mM EDTA, 0.1% (v/v) ethanol, 1 mM MgCl₂. The gradients were centrifuged

for 12–14 h at 4°C at 28000 rpm in a Beckman SW28 rotor. Gradients were fractionated as described (Taylor *et al.* 1996) and fractions used directly for enzyme assays and SDS-PAGE or frozen immediately in liquid N₂ for subsequent analysis. For protease protection experiments, peroxisomal fractions were taken from freshly run gradients and divided into three equivalent aliquots. One aliquot remained on ice with no further treatment; one aliquot was treated with 0.01 mg ml⁻¹ thermolysin on ice; and one aliquot was treated with 0.01 mg ml⁻¹ thermolysin in the presence of 0.5% v/v Triton X-100. At the end of the incubation EDTA was added to all aliquots, which were then processed for SDS-PAGE and immunodetection of ICL.

For testing ICL knockout strains, potassium acetate media contained 2% (w/v) potassium acetate, 1% (w/v) ammonium sulphate, 0.67% (w/v) yeast nitrogen base without amino acids and ammonium sulphate, 2% (w/v) Difco bacto-agar. Ethanol medium contained 0.67% (w/v) yeast nitrogen base as above, 1% (w/v) ammonium sulphate 1% (v/v) ethanol.

Yeast two-hybrid study

Yeast two-hybrid experiments were carried out as described by Lametschwandtner *et al.* (1998). β -Galactosidase was measured using o-nitrophenyl β -D-galactopyranoside (ONPG) as the substrate, the units were defined as nmol of ONPG hydrolysed per min (Miller 1972). Two independent transformants harbouring each of the respective plasmids were tested in duplicate, and the β -galactosidase activities presented were the average of four determinations (SD < 15%).

Other methods

Transformation of yeast with plasmids, protein extraction, electroblotting, and immunodetection and enzyme assays were performed as described by Taylor *et al.* (1996). The anti-castor bean ICL antibody is described in Martin and Northcote (1982); the anti-*S. cerevisiae* thiolase in Erdmann and Kunau (1994).

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