Peroxisomal targeting signals—the end and the beginning

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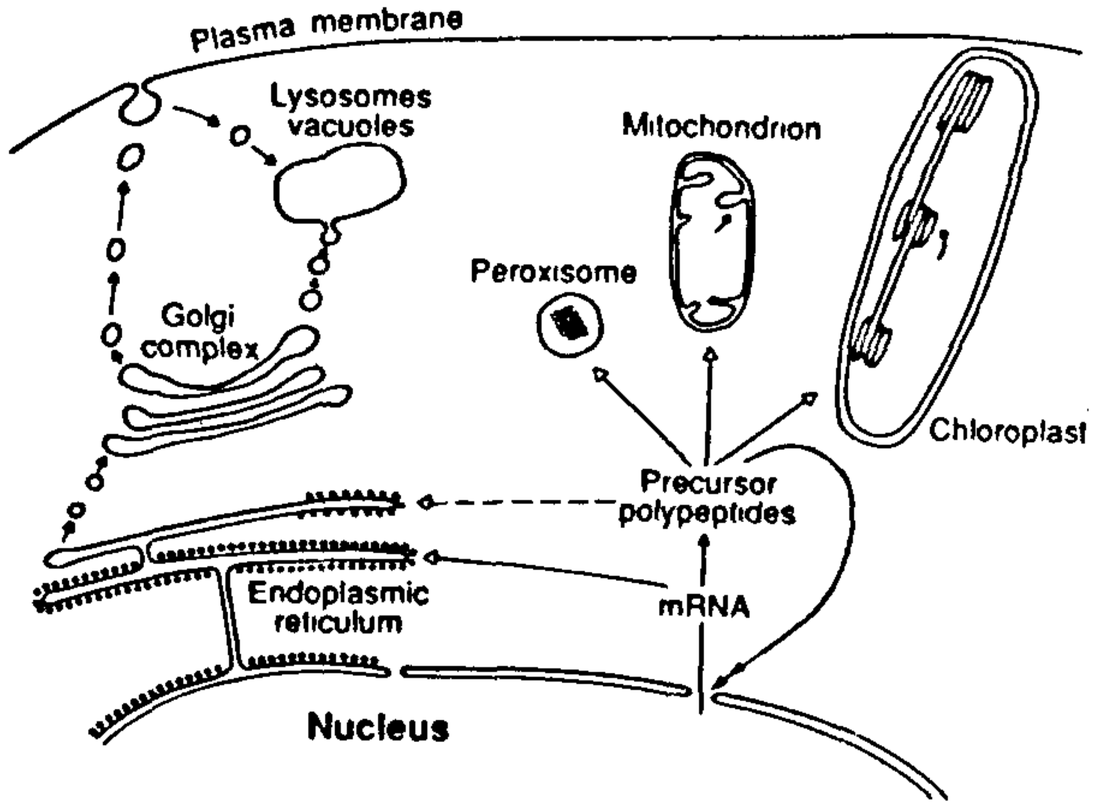
Specific signals sort proteins into their respective compartments in the cell. The targeting of most peroxisomal proteins involves a three-amino-acid sequence in the C-terminus of these proteins. Other, alternative signals may exist.

The existence of distinct subcellular compartments in all eukaryotic cells requires that proteins synthesized in the cytoplasm be sorted to their appropriate destinations where they can accomplish specific biochemical functions. For newly synthesized proteins, this sorting to subcellular compartments is achieved by two general routes (see figure). The first pathway is a cotranslational one in which proteins containing specific signals are synthesized on membrane-bound polysomes and inserted into or translocated across the membrane of the endoplasmic reticulum (ER). Other signals or chemical modifications target or retain these proteins within the membrane or lumen of ER[1-4], the Golgi[5] or the lysosome[6]. Proteins devoid of these additional signals ride the default pathway, which destines them to the plasma membrane or to the exterior of the cell[7]. The second pathway is one in which proteins synthesized on cytoplasmic polysomes are post-translationally sorted in a targeting-signal-dependent fashion to organelles such as peroxisomes[8,9], mitochondria[10], chloroplasts[11] and the nucleus[12,13]. The emphasis of this article will be on the signals that direct proteins into peroxisomes, and I concentrate primarily on the work of my own group rather than providing a global review of the field.

Importance of peroxisomes

All eukaryotes, with the exception of archaezoa, possess peroxisomes. These are single-membrane-bound organelles that encompass an electron-dense core. The organelle derives its name from the fact that enzymes that produce and degrade hydrogen peroxide (H_2O_2) are localized within this compartment. Thus they contain many H_2O_2-producing flavin oxidases and the enzyme catalase, which decomposes H_2O_2 into water and oxygen. Peroxisomes also contain all or part of several biochemical pathways. For example, they house the enzymes involved in the β-oxidation of long-chain fatty acids[14], bile-acid synthesis[15], cholesterol metabolism[16,17], plasmalogen biosynthesis[18], purine and amino-acid catabolism[19], and glyoxylate utilization[20]. Part of the problem in assigning a single predominant function to the compartment is that the menu of peroxisomal proteins varies between organisms, cell types and environments of the cell or organism.

The most convincing case for the importance of the organelle at the organismal level, however, comes from reports of the debilitating effects of peroxisomal disorders in humans[21]. These diseases are heterogeneous in the sense that a particular syndrome does not always correlate with a single genetic defect. However, they can be divided into three broad phenotypic classes: (i) those in which there is a loss of most peroxisomal functions (e.g. Zellweger syndrome[22]), (ii) diseases in which several but not all peroxisomal enzymes are absent (e.g. combined β-oxidation enzyme deficiency



Pathways for the sorting of newly synthesized proteins in eukaryotic cells. In the cotranslational pathway, proteins are synthesized on membrane-bound polysomes (solid circles), translocated into or across the membrane of the endoplasmic reticulum (ER), and subsequently retained in or sorted to ER, Golgi, lysosomes, plasma membrane or the exterior of the cell. In the post-translational pathway, proteins synthesized on free polysomes are translocated across the membranes of organelles such as peroxisomes, mitochondria or chloroplasts, or transported into the nucleus through the nuclear-pore complex.

[Picture adapted by Bart Swinkels from a diagram in Verner and Schatz[70]]

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and rhizomelic chondrodysplasia punctata[23]), and (iii) disorders caused by the loss of individual peroxisomal enzymes (e.g. thiolase deficiency[24]). Interestingly, cells from patients with Zellweger syndrome have peroxisome ghosts but lack many proteins of the peroxisomal matrix. Thus they may be deficient in the targeting of proteins into the peroxisome or in the assembly of organelles competent to translocate proteins into the matrix[25].

Patients suffering from diseases such as Zellweger syndrome eventually succumb to the disease early in childhood. They display cerebral, hepatic, ocular, renal, adrenal and skeletal abnormalities. The biochemical manifestation of the disease is the accumulation of very-long-chain fatty acids, bile-acid intermediates, and phytanic and pipecolic acids[21], and the absence of plasmalogens (alkyl-ether phospholipids), which protect cells from damage by free radicals and singlet oxygen[26,27].

The value of serendipity—luciferase is a peroxisomal protein

My group's interest in the peroxisomal-sorting problem was born out of the convergence of the three quintessential ingredients that make science exciting—excellent colleagues, serendipity and good fortune. While we were developing firefly (*Photinus pyralis*) luciferase as a reporter for gene expression[28], we performed an immunofluorescence experiment, which, in retrospect, was totally unnecessary for what we were doing at that time[29]. Indirect immunofluorescence on luciferase transiently expressed in monkey kidney cells revealed that it was in punctate, vesicular structures in the cytoplasm[29]. Since the labelling pattern was similar to that which Gilbert Keller had seen for catalase (a peroxisomal enzyme), double-indirect-immunofluorescence experiments were undertaken to show that luciferase co-localized with a known peroxisomal enzyme. We also found, using immunocryoelectron microscopy, that luciferase was peroxisomal in the lantern organ of the firefly[29]. Subsequent work revealed that this protein is peroxisomal also when expressed in yeast (*Saccharomyces cerevisiae*), plants (*Nicotiana tabacum*) and frog (*Xenopus laevis*) cells[30,31].

Unlike mitochondria and chloroplasts, which contain DNA and can encode some of their own proteins, peroxisomes are devoid of nucleic acid and must therefore import all their proteins. Our discovery that luciferase was peroxisomal in diverse eukaryotes implied that luciferase had a targeting signal that directed it to peroxisomes.

Many peroxisomal matrix proteins and several membrane proteins were known to be translocated post-translationally into the organelle (reviewed in ref. 32). Furthermore, most peroxisomal proteins are not chemically modified or proteolytically cleaved either during or after import into the organelle. Thus the nature and location of the peroxisomal targeting signal (PTS) were unknown, thereby opening the door to the use of luciferase as a model system for the identification of the first PTS.

The targeting signal

The end justifies the means

Use of the double-indirect-immunofluorescence technique to localize proteins encoded by deletion and linker-insertion mutants of luciferase revealed that two regions of the protein were necessary for peroxisomal targeting[33]. Region I encompassed about half of the 550-amino-acid protein, from amino acid 47 to amino acid 261, and region II included the C-terminal 12 amino acids. The insertion of a linker encoding four in-frame amino acids, at a variety of locations in region I, or the deletion of region II resulted in cytosolic localization of the mutant proteins[33]. Since signals for protein targeting to other compartments are relatively short polypeptide segments, we presumed that alterations in region I were probably masking the accessibility of the C-terminal PTS, which therefore became the focus of our work.

The C-terminal 12 amino acids were not only necessary but also completely sufficient for peroxisomal targeting because the fusion of these 12 amino acids onto the C-terminus of a cytosolic passenger protein, chloramphenicol acetyltransferase (CAT), resulted in peroxisomal localization of the fusion protein[33]. Examination of the sequences of proteins encoded by engineered deletion mutants with additional deletions within this 12-amino-acid region (Table 1) showed that the C-terminal tripeptide serine–lysine–leucine (SKL in the standard one-letter code) was necessary for peroxisomal localization of luciferase[9]. That this

Table 1. Mutants defining the minimal PTS in luciferase.

Amino-acid position in luciferase (5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 / 3 3 3 3 4 4 4 4 4 4 4 4 4 4 5 / 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0)	Plasmid name	Subcellular distribution
R-E-I-L-I-K-A-K-K-G-G-K-S-K-L	pRSVL	P
R-E-I-	pRSVLΔC12	C
R-E-I- G-G-K-S-K-L	pRSVLΔ539–544	P
R-E-I- K-S-K-L	pRSVLΔ539-546	P
R-E-I- S-K-L	pRSVLΔ539-547	P
R-E-I- K-L	pRSVLΔ539 548	C
R E-I- L	pRSVLΔ539-549	C
R E-I-L-I-K A-K-K-G-G K	pRSVLΔC3	C
R E-I-L-I-K-A-K K G-G K-S	pRSVLΔC2	C
R E-I L-I K A K K G G K S K	pRSVLΔC1	C
R-E I-L-I-K-A K K G-G K S K-L-S	pRSVL + S	C
R E-I L-I-K-A-K K-G G-K-S K L-I L	pRSVL + IL	C

The numbers in the table head (left) refer to the amino acid positions (read 536 etc) of the 550 amino acid long luciferase. Below are the amino-acid sequences of each mutant, the name of each mutant to its right, and, on the extreme right, the location of the protein within the cell (P, peroxisomal; C, cytosolic). [From Gould *et al*[9]]

tripeptide was also sufficient for peroxisomal targeting was deduced from the peroxisomal localization of the CAT-SKL fusion protein[9]. The same tripeptide was also found to be necessary for peroxisomal localization of luciferase in *S. cerevisiae*[34]. Thus the ability of this tripeptide to act as a PTS has been conserved in evolution.

The end is the rule

The ubiquity of N-terminal targeting signals for protein sorting to ER, mitochondria and chloroplasts raised the possibility that the C-terminal PTS in luciferase might be the exception rather than the rule. Polypeptides derived from fusions between CAT and the last 15 amino acids of the rat peroxisomal bifunctional enzyme, 15 amino acids of the rat acyl-CoA oxidase, 14 amino acids of pig D-amino-acid oxidase, 27 amino acids of human catalase, and 12 amino acids of yeast *Candida boidinii* PMP-20 were all localized to peroxisomes in monkey kidney cells, as judged by double indirect immunofluorescence[8]. Thus the C-terminal location of PTS is a common feature of many peroxisomal proteins.

The consensus tripeptide

Mutation of each of the last three amino acids of luciferase, followed by analysis of the subcellular localization of the mutant proteins has demonstrated that in the first position of the tripeptide serine is as good as alanine but these are better than lysine (which still works); in the second position lysine is as good as arginine but these are better than histidine; and in the last position leucine works more efficiently than methionine (ref. 9; and Swinkels, Gould and Subramani, unpublished). Interestingly, with the exception of human catalase (which contains a serine–histidine–leucine (SHL) sequence 10 amino acids from the C-terminus), all of the proteins identified as having a functional PTS in the previous paragraph do indeed contain the consensus tripeptide PTS at the C-terminus.

Evolutionary conservation

An antibody raised against a 12-amino-acid peptide ending in the sequence SKL was found to have remarkable specificity for the SKL–COOH sequence and did not recognize this tripeptide at internal locations in proteins. It specifically recognized peroxisomes in mammalian cells, as judged by indirect immunofluorescence and immunocryoelectron microscopy. Additionally, in Western blots of proteins from different subcellular fractions, it recognized 15–20 proteins (40% of the total Coomassie-stained peroxisomal proteins) in the peroxisome fraction and few, if any, of the proteins unique to other subcellular fractions[35]. The antibody also unambiguously labelled peroxisomes from the yeast *Pichia pastoris* and from tobacco plants[36]. The results with the antibody provide independent immunochemical evidence for the widespread use of the tripeptide PTS.

A more general signal

The term microbody has been used to describe a variety of single-membrane-bound organelles such as peroxisomes, glyoxysomes and glycosomes[37]. Though these organelles are believed to be derived from a common ancestral organelle, their constituents vary depending on the cell type or organism. They contain enzymes of the fatty-acid β-oxidation pathway in common. In addition, the glyoxysomes of plants contain enzymes of the glyoxylate pathway[20], and the glycosomes of trypanosomes contain enzymes involved in plasmalogen biosynthesis, glycolysis and β-oxidation[38].

Immunocryoelectron microscopy of cells from organisms containing these different forms of microbodies revealed that the anti-SKL antibody labelled the matrix of glyoxysomes in *Neurospora crassa* and castor-bean seedlings, and the glycosomes of *Trypanosoma brucei*[36]. This analysis was confirmed by the finding that several proteins (20–40%) in the purified organelle fractions from these organisms were labelled specifically with the anti-SKL antibody[36]. This result argues that these organelles contain, in the matrix, several proteins ending in the sequence SKL, and that microbodies must be evolutionarily related if they use the same targeting signal and, perhaps, mechanism for protein targeting. Recent evidence from experiments involving transformation of *T. brucei* with DNA constructs expressing CAT and CAT–SKL corroborates the notion that the SKL tripeptide can indeed target proteins to glycosomes[39]. In addition, some endogenous glycosomal proteins ending in the sequences AKL (alanine–lysine–leucine) and SHL have also been described[40,41]. In view of the results described above, we prefer to call the C-terminal tripeptide targeting signal a general microbody targeting signal (McTS).

Sequence analyis of known microbody proteins confirms that the C-terminal McTS is conserved at the C-terminus of at least 26 microbody matrix proteins from evolutionarily diverse organisms (Table 2). The C-terminal location of the tripeptide targeting signal also predicts that the transport of these proteins into the organelles must be post-translational.

The only organism tested in which the anti-SKL antibody did not exhibit any labelling was *Candida tropicalis*[36]. This observation and the absence of McTS at the C-terminus of many peroxisomal proteins from *Candida* sp.[42] raised the possibility that *Candida* sp. might represent an exception to the extraordinary

Table 2. Conservation of the tripeptide PTS in microbody proteins.

Protein	Total amino acids	Conserved amino acids	Location C-terminal	Reference
Rat acyl-CoA oxidase	661	Ser–Lys–Leu	+	52
Rat bifunctional enzyme	772	Ser–Lys–Leu	+	53
Rat sterol carrier protein 2	143	Ala–Lys–Leu	+	54
Pig D-amino-acid oxidase	347	Ser–His–Leu	+	55
P. pyralis luciferase	550	Ser–Lys–Leu	+	28
P. plagiophthalamus luciferase	543	Ser–Lys–Leu	+	56
L. cruciata luciferase	548	Ala–Lys–Met	+	57
Cucumis sativus malate synthase	568	Ser–Lys–Leu	+	58
Brassica napus malate synthase	561	Ser–Arg–Leu	+	59
Spinach glycolate oxidase	369	Ala–Arg–Leu	+	60
Gossypium hirsutum isocitrate lyase	576	Ala–Arg–Met	+	61
B. napus isocitrate lyase	576	Ser–Arg–Met	+	62
Ricinus communis isocitrate lyase	576	Ala–Arg–Met	+	63
S. cerevisiae trifunctional enzyme	899	Ser–Lys–Leu	+	W. Kunua, pers. comm.
S. cerevisiae citrate synthase	460	Ser–Lys–Leu	+	64
S. cerevisiae DAL7 gene product	554	Ser–Lys–Leu	+	65
C. tropicalis trifunctional enzyme	906	Ala–Lys–Ile	+	43
C. boidinii PMP-20	167	Ala–Lys–Leu	+	66
T. brucei glucose-6-phosphate isomerase	606	Ser–His–Leu	+	41
T. brucei glyceraldehyde-3-phosphate dehydrogenase	358	Ala–Lys–Leu	+	40
T. cruzi glyceraldehyde-3-phosphate dehydrogenase	359	Ala–Arg–Leu	+	67
Drosophila melanogaster uricase	352	Ser–His–Leu	+	68
Mouse uricase	304	Ser–Arg–Leu	+	68
Pig uricase	304	Ser–Arg–Leu	+	68
Baboon uricase	304	Ser–Arg–Leu	+	68
Rat uricase	303	Ser–Arg–Leu	+	69

Adapted from ref 9

evolutionary conservation of the tripeptide McTS. This explanation can be discounted, however, by the recent evidence from R. Rachubinski's laboratory, which shows that the trifunctional enzyme from *C. tropicalis* ends in the sequence AKI (alanine–lysine–isoleucine)[43] and that this sequence is necessary for its peroxisomal localization[44]. Furthermore, the last 12 amino acids of the *C. boidinii* PMP-20 protein act as PTS and are known to end in the sequence AKL, which is a version of the tripeptide McTS[8]. Therefore *Candida* sp. also uses McTS but a slightly different version of it. Similar variations have been described in the use of the C-terminal tetrapeptides KDEL (lysine–aspartic acid–glutamic acid–leucine), HDEL or DDEL as ER-retention signals in man, *S. cerevisiae* and the yeast *Kluyveromyces lactis* respectively[45].

Alternative targeting signals

The absence of labelling of microbody membranes in immunoblotting or immunoelectron-microscopy expe-riments with the anti-SKL antibody[35,36] and the absence of a C-terminal McTS in the two peroxisomal membrane proteins sequenced to date[46,47] prompt us to suggest that the tripeptide McTS may be used primarily for the targeting of peroxisomal matrix proteins; peroxisomal membrane proteins would be targeted by the use of a different signal. If true, this prediction would explain why cells from Zellweger patients have peroxisome membrane ghosts but no matrix proteins.

The exception is at the beginning

Not all peroxisomal proteins enter the organelle by the use of one type of signal. Thus, despite the high degree of conservation of McTS, other general microbody targeting signals or peroxisome-, glyoxysome- or glycosome-specific targeting signals are likely to exist. This is because several known peroxisomal proteins contain only internal SKL-like sequences but no C-terminal sequence that resembles McTS, and a few proteins, such as rat catalase, do not contain the sequence at all. To date we have no evidence that the tripeptide McTS can function when it is moved to internal locations in a cytosolic passenger protein.

In an attempt to identify other PTSs, we chose rat thiolase as a model. This protein, which is involved in the β-oxidation of fatty acids, exists in at least two peroxisomal forms[48,49] and also has a mitochondrial homologue[50]. Unlike most other peroxisomal proteins, the peroxisomal thiolases have 26- and 36-amino-acid leader peptides respectively, which are cleaved following import into peroxisomes[48,49]. In contrast, the mito-chondrial form is peculiar in that it has an amino-terminal mitochondrial targeting signal that is not cleaved[50]. We have found recently that a new PTS exists in the cleaved amino-terminal leaders of the two peroxisomal thiolases[51]. The existence of two distinct types of targeting signals for organellar transport of proteins is not unprecedented. The ADP/ATP-carrier protein and the β-subunit of the F_0/F_1-ATPase have different mitochondrial targeting signals[10].

Summary

Starting from a serendipitous observation we are indeed fortunate to have come so far in the elucidation of the signals that sort proteins into peroxisomes. Our own focus has shifted to elucidation of the mechanisms involved in peroxisomal transport. It will be interesting to see whether the transport of proteins across the peroxisomal membrane can serve as an experimental paradigm for protein translocation across other cellular membranes.

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