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Topical Review

Targeting of Proteins into the Peroxisomal Matrix

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Introduction

One of the hallmarks of all eukaryotic cells is the existence of distinct subcellular compartments into which proteins must be sorted for the segregation of biochemical functions. This segregation is achieved by endowing proteins with specific amino acid sequences or chemical modifications and by the selective action of sorting machineries which recognize these targeting signals and direct the subsequent traffic of proteins to their final destinations. Proteins encoded by nuclear genes are either retained in the cytosol because they lack specific targeting signals or are routed along two generalized pathways. The first of these is the cotranslational pathway in which proteins containing specific signals are synthesized on membrane-bound polysomes and are inserted into or translocated across the membrane of endoplasmic reticulum (ER). Following this, additional signals or protein modifications govern the retention of these proteins within the membrane or the lumen of the ER (Kuroki, Russnak & Ganem, 1989; Nillson, Jackson & Peterson, 1989; Pelham, 1989; Stirzaker & Both, 1989), or their transport further to the Golgi (Machamer & Rose, 1987) or lysosome (Kornfeld & Mellman, 1989). In the absence of such additional signals, proteins traverse the default pathway (Pfeffer & Rothman, 1987), which directs them to the plasma membrane or to the exterior of the cell. The alternative pathway is one in which proteins synthesized on free cytoplasmic polysomes are post-translationally routed in a targeting signal-dependent manner to compartments such as the peroxisome (Gould, Keller & Subramani, 1988; Gould et al., 1989), mitochondrion (Attardi & Schatz, 1988), chloroplast (Smeekens, Weisbeek & Robinson, 1990) or the nucleus (Colledge et al., 1986; Richardson, Roberts & Smith, 1986). The focus of this review will be on the signals that sort proteins into peroxisomes. It is not meant to be a comprehensive review of the more general problem of the mechanism of protein translocation into peroxisomes.

Peroxisomes are ubiquitous single-membrane bound organelles that perform many critical functions. They are found in all eukaryotes except archaezoa (Cavalier-Smith, 1987). They house a variety of H_2O_2-producing flavin oxidases and catalase which decomposes H_2O_2 into oxygen and water. In addition, they contain enzymes involved in plasmalogen biosynthesis (Hajra & Bishop, 1982), the β-oxidation of long-chain fatty acids (Lazarow & de Duve, 1976), bile acid synthesis (Krisans et al., 1985), cholesterol metabolism (Thompson et al., 1987; Keller et al., 1989; Thompson & Krisans, 1990), purine and amino-acid catabolism (Takada & Noguchi, 1986), and glyoxylate utilization (Briedenbach & Beevers, 1967). The specific repertoire of peroxisomal enzymes is often dependent upon the organism, tissue or the environmental milieu of the cell or organism.

The plight of humans with debilitating peroxisomal disorders highlights the importance of the organelle particularly at the organismal level (Wanders et al., 1988). These diseases fall into three classes: (1) Those resulting in a generalized loss of peroxisomal functions (e.g., Zellweger's syndrome; Zellweger, 1988); (2) diseases in which several but not all peroxisomal enzymes are absent (e.g. combined β-oxidation enzyme deficiency and Rhizomelic chondrodysplasia punctata, Schutgens et al., 1988); and (3) disorders resulting from the loss of individual peroxisomal enzymes (e.g. thiolase deficiency; Schram et al., 1987). While the genetic basis of most of these disorders remains an enigma, there is evidence that the generalized disorder may manifest itself due to the failure of protein translocation into peroxisomes (Schram et al., 1986). Consequently, some proteins such as catalase accumulate in the cytosol while

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others such as some of the β-oxidation enzymes are rapidly turned over (Wanders et al., 1984). Somatic cell fusion experiments have placed cells from patients with peroxisomal disorders into at least six complementation groups (Roscher et al., 1989).

The medical prognosis for some of the more severe diseases such as Zellweger's syndrome is rather bleak (Zellweger, 1988). These patients display many cerebral, hepatic, ocular, renal, adrenal and skeletal abnormalities which result in death within a few years after birth. The loss of peroxisome function is apparent at the biochemical level by the accumulation of very long-chain fatty acids, bile acid intermediates, phytanic and pipecolic acid (Wanders et al., 1988) and by the absence of ether phospholipids which protect proteins and lipids from damage by free radicals and singlet oxygen (Morand et al., 1988; Zoeller, Morand & Raetz, 1988).

Unlike mitochondria and chloroplasts which contain DNA and can encode several of their own proteins, peroxisomes are devoid of nucleic acid and must therefore import all their proteins which are encoded by nuclear genes. Many peroxisomal matrix proteins and several membrane proteins (reviewed by Gould & Subramani, 1991) are known to be translocated post-translationally into the organelle. While most peroxisomal proteins are not modified chemically or proteolytically either during or after import into the organelle, a few such as acyl-CoA oxidase and thiolase are proteolytically cleaved following import (Fujiki & Lazarow, 1985). However, this cleavage is not essential for protein translocation into the organelle (Balfe et al., 1990).

Firefly Luciferase is Targeted into Peroxisomes of Diverse Eukaryotes from Yeasts to Mammals

The initial observation that the firefly (*Photinus pyralis*) luciferase was a peroxisomal enzyme came from an unexpected and serendipitous observation made during the development of this bioluminescent enzyme as a reporter for gene expression in my lab (deWet et al., 1987). Indirect immunofluorescence on luciferase transiently expressed in monkey kidney cells revealed that it was localized to punctate vesicular structures in the cytoplasm (Keller et al., 1987). Double indirect immunofluorescence experiments demonstrated that luciferase colocalized in these cells with catalase, a *bona fide* peroxisomal enzyme (Keller et al., 1987), thus providing the first evidence that luciferase was a peroxisomal protein. Subsequent work showed that luciferase was also targeted into peroxisomes when expressed in insect (*Photinus pyralis*), yeast (*Saccharomyces cerevisiae*), plant (*Nicotiana tabacum*), frog (*Xenopus laevis*) or mammalian cells (Keller et al., 1987; Gould et al., 1990*a*; Holt Garlick & Cornel, 1990). The availability of the cloned cDNA encoding firefly luciferase and the absence of cross-reactive proteins in these organisms (except the firefly) provided an excellent model system for the elucidation of the peroxisomal targeting signal in the protein by determination of the subcellular localization of mutant luciferases using indirect immunofluorescence or immunoelectron microscopy.

A C-Terminal Tripeptide is the Peroxisomal Targeting Signal of Luciferase

Analysis of the subcellular localization of proteins encoded by deletion and linker-insertion mutants of luciferase in monkey kidney cells demonstrated that two regions within the protein were necessary for peroxisomal import. The first was a broad region between amino acids 47-261 of the 550 amino acid protein, while the second corresponded to the C-terminal 12 amino acids (aa 539-550). The insertion, into the first region, of a linker encoding four amino acids in frame with the rest of the protein, or the deletion of the C-terminal 12 amino acids from the second region, resulted in the cytoplasmic localization of the proteins (Gould, Keller & Subramani, 1987). Reasoning that the mutations in the first region were probably altering the conformation of the protein and the accessibility of the true PTS, we focussed our attention on the C-terminal segment of luciferase which was necessary for peroxisomal import.

Fusion of the last 12 amino acids of luciferase onto the C-terminus of a cytosolic passenger protein, chloramphenicol acetyltransferase (CAT) resulted in the transport of the fusion protein into peroxisomes (Gould et al., 1987). Finally, further analysis of the localization of proteins encoded by mutants containing deletions within these 12 amino acids of luciferase showed that the C-terminal tripeptide (SKL) was the PTS. Remarkably, this simple tripeptide was also completely sufficient to transport CAT into peroxisomes, when fused onto its C-terminus (Gould et al. 1989).

Since luciferase was targeted to peroxisomes in many species, the luciferase mutants were expressed in *S. cerevisiae* and their subcellular localization was determined by immunocryoelectron microscopy. The same tripeptide was also necessary for peroxisomal targeting of luciferase in yeast, suggesting that the PTS has been conserved in evolution (Distel et al.[1]).

[1] Distel, B., Gould, S.J., Voorn-Brouwer, T., van der Berg, M., Tabak, H.F., Subramani, S. 1991. (*submitted*)

The Analysis of PTS Variants Identifies a Consensus PTS

Each amino acid of the tripeptide SKL in luciferase was mutated to a variety of other amino acids. These mutant proteins were then localized in mammalian cells as described before. The results demonstrated that Ser could be substituted by Ala or Cys; Lys by His or Arg and Leu by Met, without affecting peroxisomal targeting (Gould et al., 1989; B.W. Swinkels, S.J. Gould and S. Subramani, *unpublished*).

Existence of a Functional PTS at the C-Termini of Many Peroxisomal Proteins

Because the sorting of proteins to the mitochondria, chloroplasts and the ER generally involves the use of amino-terminal signals, the unusual C-terminal PTS in luciferase led us to question the generality of the C-terminal signal in the peroxisomal transport of proteins. Fusions between CAT and the C-termini of five other peroxisomal proteins were expressed in monkey kidney cells and tested for their subcellular localization using indirect immunofluorescence. In each case the fusion protein was translocated into peroxisomes. These results demonstrate that 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 *Candida boidinii* PMP-20 contain a C-terminal PTS (Gould et al., 1988). Miyazawa et al. (1989) have provided independent evidence that rat acyl-CoA oxidase contains a C-terminal sequence that is necessary and sufficient for localization into rat liver peroxisomes in vitro. Thus the C-terminal location of the PTS is indeed a general feature of many peroxisomal proteins. With the exception of catalase, all of these proteins contain a C-terminal tripeptide similar to the one in luciferase. The human catalase contains the tripeptide SHL about 10 amino acids in from the C-terminus. It is not clear whether this or some other sequence within the last 27 amino acids of catalase actually functions as the PTS.

The Ability of the Tripeptide PTS to Function is Context Dependent

Indirect evidence suggests that the tripeptide PTS is context dependent in its ability to be recognized. Linker insertions within the N-terminal half of luciferase, as well as large fusions between cytosolic passenger proteins and luciferase produce polypeptides which fail to be transported into peroxisomes, even though the PTS should have been present at the C-terminus of these proteins (Gould et al., 1987). The simplest explanation for this result is that the PTS must be accessible in the folded protein. This context dependence is not unprecedented and has been documented for the targeting of proteins to the nucleus (Roberts, Richardson & Smith, 1987).

Antibodies to the SKL Tripeptide Recognize Many Peroxisomal Matrix Proteins in Diverse Species

Indirect immunofluorescence and immunocryoelectron microscopy experiments were used to demonstrate the specific recognition of peroxisomes in mammalian cells by an antibody raised against a peptide ending in the sequence SKL (Gould et al., 1990*b*). This antibody has a remarkable specificity for the SKL-COOH sequence and recognizes it only when the tripeptide is at the carboxy-terminus of proteins and not when the SKL is located internally in proteins. Western blots of proteins from different subcellular fractions confirmed that 15–20 proteins (40% of the total Coomassie blue-stained peroxisomal proteins) and few, if any, of the unique proteins from other subcellular fractions were recognized by this antibody. Peroxisomes of the yeast *Pichia pastoris* and tobacco plants were also recognized by the antibody (Keller et al., 1991). These data provide independent immunological evidence that the tripeptide PTS is highly conserved in evolution and that it functions as a major PTS.

The Tripeptide PTS Is Really a General Microbody Targeting Signal

Organelles such as peroxisomes, glyoxysomes and glycosomes have been referred to more broadly as microbodies, which were first described morphologically by Rhodin (1954) as a single-membrane-bound organelle with an electron dense matrix. One common biochemical feature of these organelles is that they posses the enzymes involved in the β-oxidation of fatty acids. However, they differ in their other constituents, depending on the organism or cell type from which they are derived. While the peroxisomes also contain hydrogen-peroxide generating oxidases and catalase (in addition to the other proteins described earlier), the glyoxysomes are unique in that they contain some or all of the enzymes of the glyoxylate pathway and the glycosomes contain the glycolytic enzymes.

Immunocryoelectron microscopy with the anti-SKL antibody showed specific labeling of the matrix

of glyoxysomes in *N. crassa* and castor bean seedlings, as well as in the glycosomes of *Trypanosoma brucei* (Keller et al., 1991). Western blot analysis of proteins in purified microbody fractions from each of these organisms revealed many proteins ending in the SKL tripeptide. This result suggests strongly that the SKL tripeptide is used as a general signal to target proteins to microbodies. In each of these organelles, 20–40% of the Coomassie-stained proteins were recognized by the anti-SKL antibody. One important implication of this result is that peroxisomes, glyoxysomes and glycosomes must be evolutionarily related if they use the same signal and perhaps mechanism for protein targeting. Therefore, it would be more accurate to refer to the C-terminal tripeptide PTS as a general microbody targeting signal.

The C-Terminal Microbody Targeting Signal Is Extremely Well Conserved during Evolution

Analysis of the protein sequences of known microbody proteins confirms that the C-terminal consensus microbody targeting signal is present in at least 26 microbody matrix proteins from evolutionarily diverse organisms (Table). Interestingly, the consensus tripeptide is located less frequently at the C-termini of nonperoxisomal proteins than would be expected by chance. The only other proteins that contain such sequences at their C-termini are a few encoded by bacteria or mitochondria and a few that are located in nonperoxisomal membranes or are secreted from cells (Gould et al., 1988, 1989). None of these would be mistargeted to peroxisomes because proteins synthesized by mitochondrial or bacterial genomes never encounter peroxisomes, and proteins entering the ER/secretory pathway engage themselves in the cotranslational pathway before the C-terminus containing the microbody targeting signal is synthesized. The C-terminal location of the tripeptide microbody targeting signal also provides a very satisfying explanation for the post-translational mode of transport of proteins into peroxisomes.

Two internal PTSs have been described in acyl-CoA oxidase from *C. tropicalis* by Small et al. (1987). The absence of the consensus tripeptide PTS in these two segments and at the C-terminus of several peroxisomal proteins from *Candida* sp. (Small & Lewin, 1990), as well as our inability to detect any immunolabeling of *C. tropicalis* peroxisomes with the anti-SKL antibody (Keller et al., 1991), raise the possibility that *Candida* sp. might represent an exception to the evolutionary conservation of the tripeptide microbody targeting signal. However, this is unlikely for several reasons: (1) The last 12 amino acids of the *C. boidinii* PMP-20 gene contain a PTS ending in AKL which is indeed a version of the tripeptide microbody targeting signal; (2) Recent evidence from Richard Rachubinski's lab shows that the *C. tropicalis* trifunctional enzyme ends in the sequence AKI (Nuttley, Aitchison & Rachubinski, 1988) and that this is necessary for peroxisomal localization (Aitchison, Murray & Rachubinski, 1991). These results suggest that *Candida* sp. do indeed use a slightly different version of the consensus tripeptide microbody targeting signal. Analogous variations can be found in the use of the C-terminal tetrapeptide KDEL, HDEL and DDEL as ER retention signals in humans, *S. cerevisiae* and *Kluyveromyces lactis*, respectively (Lewis, Sweet & Pelham, 1990).

Peroxisomal Membrane Proteins May Use Different Targeting Signals

It is striking that both immunoblotting experiments with peroxisomal membrane fractions from rat liver (Gould et al., 1990*b*) and immunoelectron microscopy of microbodies from several organisms (Keller et al., 1991) with the anti-SKL antibody failed to reveal any membrane proteins that were recognized by the antibody. The tripeptide microbody targeting signal is therefore used principally for the targeting of peroxisomal matrix proteins into the organelle. Peroxisomal membrane proteins are probably targeted to the organelle by the use of some other signal. The sequences of one peroxisomal membrane protein from *C. boidinii* (PMP-47, McCammon et al., 1990) and two from mammalian sources (70 kD protein, Kamijo et al., 1990; 35 kD protein, Tsukamoto, Miura & Fujiki, 1991) have been published, but none of these has a C-terminal SKL or SKL-like sequence.

Multiple Signals Involved in the Targeting of Peroxisomal Matrix Proteins

Despite the remarkable conservation and widespread use of the C-terminal microbody targeting signal, it is very likely that other general microbody targeting signals or peroxisome-, glyoxysome- or glycosome-specific targeting signals exist. An examination of the amino acid sequences of known microbody proteins shows that while many do indeed contain the tripeptide targeting signal at their C-terminii, there are also many exceptions (Gould et al, 1989). Most of these contain the tripeptide signal at internal locations but a few, such as rat catalase, do not contain the signal at all. The absence of evidence to suggest that the tripeptide microbody targeting

Table. Conservation of the tripeptide targeting signal in microbody proteins

Protein	Total # aa	Conserved aa	Location C-terminal	Reference
Rat acyl-CoA oxidase	661	Ser-Lys-Leu	+	Miyazawa et al., 1987
Rat bifunctional enzyme	772	Ser-Lys-Leu	+	Osumi et al., 1985
Rat sterol carrier protein 2	143	Ala-Lys-Leu	+	Billheimer et al., 1990
Pig D-amino acid oxidase	347	Ser-His-Leu	+	Ronchi et al., 1982
P. pyralis luciferase	550	Ser-Lys-Leu	+	de Wet et al., 1987
P. plagiophthalamus luciferase	543	Ser-Lys-Leu	+	Wood et al., 1989
Luciola cruciata luciferase	548	Ala-Lys-Met	+	Masuda et al., 1989
Cucumis sativus malate synthase	568	Ser-Lys-Leu	+	Smith & Leaver, 1986
Brassica napus malate synthase	561	Ser-Arg-Leu	+	Comai et al., 1989*a*
Spinach glycolate oxidase	369	Ala-Arg-Leu	+	Volokita & Somerville, 1987
Gossypium hirsutum isocitrate lyase	576	Ala-Arg-Met	+	Turley et al., 1990
B. napus isocitrate lyase	576	Ser-Arg-Met	+	Comai et al., 1989*b*
Ricinus communis isocitrate lyase	576	Ala-Arg-Met	+	Beeching & Northcote, 1987
S. cerevisiae trifunctional enzyme	899	Ser-Lys-Leu	+	W. Kunau, *personal communication*
S. cerevisiae citrate synthase2	460	Ser-Lys-Leu	+	Lewin et al., 1990
S. cerevisiae DAL7 gene product	554	Ser-Lys-Leu	+	Yoo & Cooper, 1989
C. tropicalis trifunctional enzyme	906	Ala-Lys-Ile	+	Nuttley et al., 1988
C. boidinii PMP-20	167	Ala-Lys-Leu	+	Garrard & Goodman, 1989
T. brucei glucose-6-phosphate isomerase	606	Ser-His-Leu	+	Marchand et al., 1989
T. brucei glyceraldehyde-3-phosphate dehydrogenase	358	Ala-Lys-Leu	+	Michels et al., 1986
T. cruzi glyceraldehyde-3-phosphate dehydrogenase	359	Ala-Arg-Leu	+	Kendall et al., 1990
Drosophila melanogaster uricase	352	Ser-His-Leu	+	Wallrath et al., 1990
Mouse uricase	304	Ser-Arg-Leu	+	"
Pig uricase	304	Ser-Arg-Leu	+	"
Baboon uricase	304	Ser-Arg-Leu	+	"
Rat uricase	303	Ser-Arg-Leu	+	Alvares et al., 1989

Adapted from Gould et al., 1989.

signal can function at interval locations in proteins (Gould et al., 1988), and the presence of the tripeptide at interval locations in many nonperoxisomal proteins argue quite strongly that other targeting signals must exist.

Our own work on rat thiolase has led to the identification of a new PTS that can function at internal locations (Swinkels et al., 1991). Thus mammalian cells use multiple PTSs. The recognition of several glycosomal proteins by the anti-SKL antibody (Keller et al., 1991), the transport of the CAT-SKL fusion protein into glycosomes (Fung & Clayton, 1991), and the ability of a different C-terminal 21 amino acid extension from the glycosomal phosphoglycerate kinase to function as a glycosomal targeting signal (Swinkels, Evers & Borst, 1988; Fung & Clayton, 1991) suggest that multiple signals may also be involved in glycosomal targeting of proteins.

Though the discovery of multiple peroxisomal targeting signals is not entirely unexpected, it is particularly important in the elucidation of the protein targeting defect in Zellweger syndrome patients. Until recently, Zellweger syndrome patients were believed to be defective in the transport of most, if not all, matrix proteins into the organelle (Santos et al., 1988). However, Balfe et al. (1990) have described peroxisomal membrane ghosts in certain Zellweger patients that import the thiolase precursor into the peroxisomes but fail to import other proteins such as acyl-CoA oxidase and catalase. Thus the cells from these patients are competent to import proteins containing one type of PTS. However, cells from certain Zellweger patients are incapable of transporting proteins with the SKL PTS, as demonstrated by the fact that microinjected luciferase is transported into peroxisomes of normal human cells but not of Zellweger cells (Walton et al., 1990). In view of these results, it seems reasonable to conjecture that the SKL and thiolase PTSs would be recognized by different receptors initially. The intriguing question then is whether these receptors interact with the same or independent translocation machineries to facilitate import.

The existence of two distinct types of targeting signals and receptors has a parallel in the import of proteins into mitochondria. The beta-subunit of the F_0/F_1 ATPase and the ADP/ATP carrier protein have distinct mitochondrial targeting signals which are proposed to bind to different receptors, MOM19 and MOM72, respectively (Pfanner & Neupert,

1990). It has been suggested that these receptors interact with common translocation proteins or general insertion proteins (GIPs) at specific sites on the mitochondrial outer membrane (Pfaller et al., 1988).

A Human Disease Caused by the Mistargeting of a Peroxisomal Protein to Mitochondria

Just as the lack of peroxisomal import appears to be responsible for a human disease such as Zellweger syndrome, there has been a recent report of a disease caused by the missorting of an essential peroxisomal protein, L-alanine : glyoxylate aminotransferase I (AGT1) (Danpure et al., 1989: Takada et al., 1990). This protein catalyzes the transamination of gloxylate to glycine using L-alanine as the donor of the amino group. The failure to detoxify glyoxylate leads to the conversion of glyoxylate to oxalate whose low solubility results in hyperoxaluria (Williams & Wandzilak, 1989).

AGT1 is unusual in that its subcellular localization is species dependent. It is peroxisomal in primates and lagomorphs, mitochondrial in carnivores and in both organelles in rodents. The deficiency of AGT1 in humans is the cause of a lethal, autosomal recessive disorder called primary hyperoxaluria type I (PH1). While most patients are devoid of enzymatic activity, about 40% of the patients have some residual activity. In about half of these, AGT1 was found to be mistargeted to mitochondria (Takada et al., 1990).

A comparison of the sequences of the rat and human AGT1 shows that in humans the putative ATG codon that could have coded for a protein with an extra 22 amino acid-mitochondrial-leader peptide is mutated to ATA. It has been suggested that the targeting defect in PH1 could be due to a polymorphism that reintroduces all or part of the mitochondrial targeting signal and a second mutation that induces a deficiency in peroxisomal import (Purdue, Takada & Danpure, 1990; Takada et al., 1990). This example underscores the importance of both subcellular compartmentalization and the fidelity of protein sorting in the genesis of human disease.

Summary

During the last few years much has been learned regarding signals that target proteins into peroxisomes. The emphasis in the near future will undoubtedly shift towards the elucidation of the mechanism of import. The use of mammalian and yeast cells deficient in peroxisome assembly and/or import (Zoeller & Raetz, 1986; Erdmann et al., 1989; Cregg et al., 1990; Morand et al., 1990; Tsukamoto, Yokota & Fujiki, 1990) should provide a handle on the genes (Erdmann et al., 1991; Tsukamoto et al., 1991) involved in these processes. This will have to be coupled with further development of in vitro systems which will permit the dissection of the steps in the translocation of proteins into peroxisomes. Though some progress has been made in the development of such assays (Imanaka et al., 1987; Small et al.,1987, 1988; Miyazawa et al., 1989), the fragility of peroxisomes and the absence of biochemical hallmarks of import (such as protein modifications or proteolytic processing) have hindered progress. Since peroxisomes exist in the form of a reticulum in mammalian cells (Gorgas, 1984), all peroxisome purification schemes (from mammalian cells at least) must undoubtedly rupture the peroxisomes, which then reseal to form vesicular structures. Additionally, the reliance on the latency of catalase alone as a major criterion for the integrity of peroxisomes ignores the fact that many other matrix proteins leak out of peroxisomes at vastly different rates during purification of the organelles (Thompson & Krisans, 1990). In view of these problems, the development of peroxisomal transport assays with semi-intact cells would also constitute an important advance. It is very likely that in the next few years we will witness some major advances in our understanding of the mechanism by which proteins enter this organelle.

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