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Peroxisomal protein import is conserved between yeast, plants, insects and mammals

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We have previously demonstrated that firefly luciferase can be imported into peroxisomes of both insect and mammalian cells. To determine whether the process of protein transport into the peroxisome is functionally similar in more widely divergent eukaryotes, the cDNA encoding firefly luciferase was expressed in both yeast and plant cells. Luciferase was translocated into peroxisomes in each type of organism. Experiments were also performed to determine whether a yeast peroxisomal protein could be transported to peroxisomes in mammalian cells. We observed that a C-terminal segment of the yeast (*Candida boidinii*) peroxisomal protein PMP20 could act as a peroxisomal targeting signal in mammalian cells. These results suggest that at least one mechanism of protein translocation into peroxisomes has been conserved throughout eukaryotic evolution.

Key words: firefly luciferase/peroxisome/translocation/yeast

Introduction

The segregation of proteins into subcellular compartments is a hallmark of eukaryotic cells. The observation that most eukaryotes possess the same sets of organelles (including peroxisomes) suggests that the common ancestor of present-day eukaryotes contained these organelles. It follows that the mechanisms of protein import into organelles were established prior to the divergence of eukaryotes. Experiments described in this report address the question of whether the signals for protein import into peroxisomes have been functionally conserved during the course of eukaryotic evolution.

The peroxisome is a small organelle bound by a single membrane and is thought to form by division or budding of pre-existing peroxisomes. Its proteins are synthesized on free polysomes and imported into the organelle post-translationally. The import process does not involve any detectable modification of the translocated protein (for a review of peroxisome biogenesis, see Lazarow and Fujiki, 1985). Firefly luciferase has been used as model protein for the elucidation of a peroxisomal targeting signal active in mammalian cells (Gould *et al.*, 1987, 1989). We have tested whether luciferase, which is peroxisomal in higher eukaryotes (Keller *et al.*, 1987), is also peroxisomal when expressed in yeast and plant cells. In addition, we asked whether a yeast peroxisomal protein, PMP20 from *Candida boidinii*, contained a signal capable of directing protein import into peroxisomes in mammalian cells.

Results

Luciferase is peroxisomal in plants

Luciferase was expressed in tobacco (*Nicotiana tabacum*) plants from a transgene containing the luciferase cDNA driven by the cauliflower mosaic virus (CaMV) 35S RNA promoter (Ow *et al.*, 1986). Leaf protoplasts were prepared from transgenic plants and used in immunocryoelectron microscopy experiments to determine the subcellular distribution of luciferase. Plant leaf peroxisomes are bound by a single membrane, often found in close association with chloroplasts, and commonly contain a crystalloid protein core. Gold particles (electron-dense dots) indicating the distribution of luciferase were found over organelles characteristic of plant peroxisomes (Figure 1A and B). Labeling was specific for peroxisomes as only few gold particles were found over the cytoplasm, chloroplasts or mitochondria.

Luciferase is peroxisomal in yeasts

The yeast *Saccharomyces cerevisiae* contains only one or a few small peroxisomes when grown under normal conditions (Parish, 1975), but peroxisomes multiply and enlarge in medium containing oleic acid (Veenhuis *et al.*, 1987). Under these conditions, *S.cerevisiae* can be used in an *in vivo* peroxisomal import assay (Distel *et al.*, 1987).

The luciferase cDNA was placed downstream of the constitutive promoter for the phosphoglycerate kinase (PGK) gene on a multicopy plasmid. The vector containing this chimeric gene, pPGK-LUC-YE23R, was transformed into *S.cerevisiae*. Expression of luciferase was confirmed by Western blot analysis (not shown). Transformed yeast cells were grown on oleic acid under conditions that induce peroxisome proliferation (Distel *et al.*, 1987). They were then fixed and prepared for immunoelectron microscopy. Lowicryl-embedded ultrathin sections of yeast cells were incubated with antibodies raised against luciferase followed by protein A/gold labeling. The oleic acid-induced peroxisomes appear as single, membrane-bound, ovoid organelles containing a slightly granular matrix (Distel *et al.*, 1987; Veenhuis *et al.*, 1987). Immunocytochemistry revealed that luciferase was present within these peroxisome-like organelles (Figure 2A and B). The labeling was specific, as very few gold particles were detected over other organelles such as the mitochondria (Figure 2A) or nucleus (Figure 2B). A double-labeling experiment using antibodies specific for

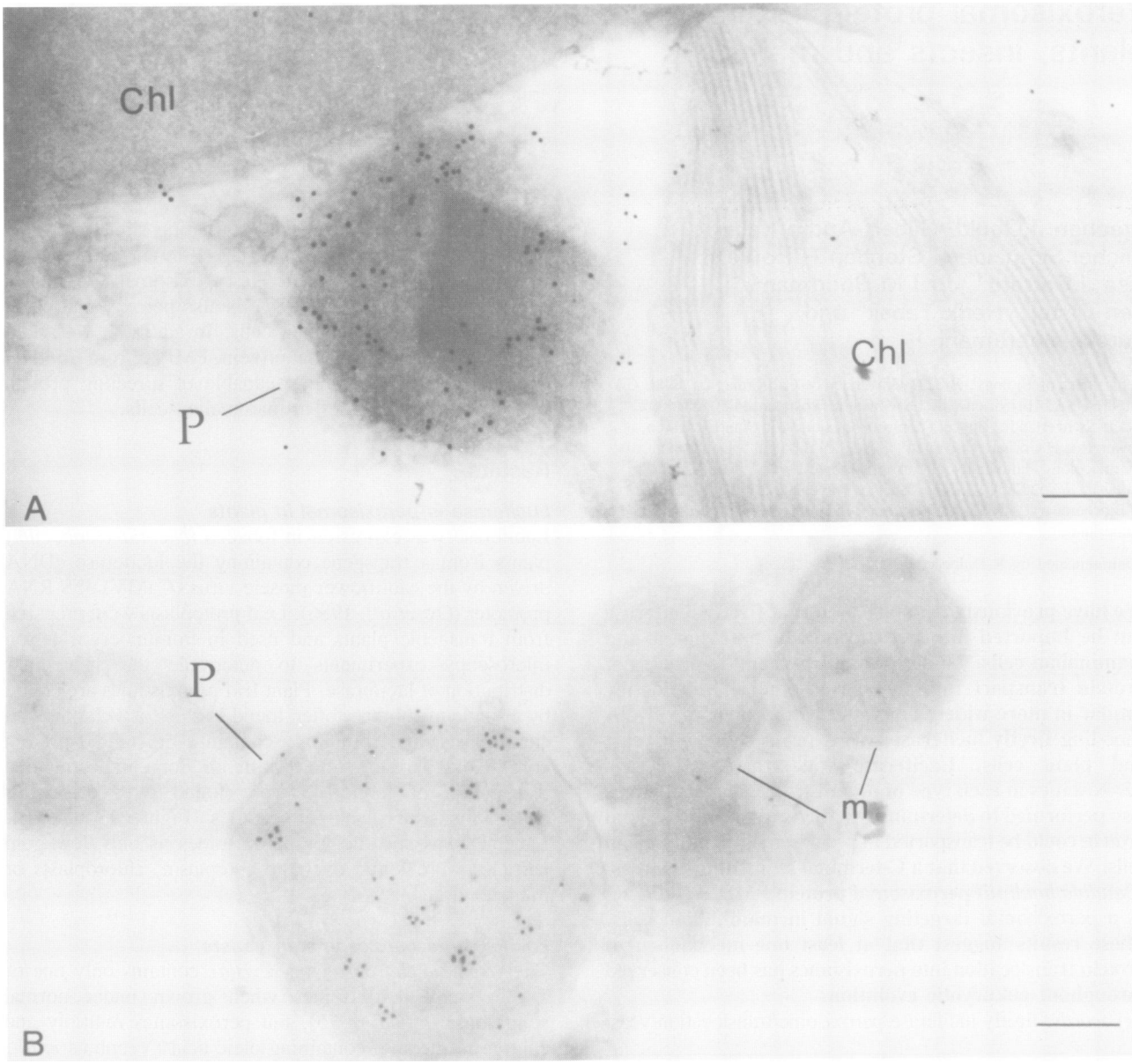

Fig. 1. Luciferase is targeted to the leaf peroxisome of transgenic *N. tabacum* Wi-38 plants that express the firefly luciferase gene. Electron-dense gold particles represent the distribution of luciferase. (**A**) Firefly luciferase is uniformly distributed in a single membrane-bound organelle wedged between the chloroplasts (Chl). The crystaloid core unambigously identifies this organelle as a peroxisome (P). No labeling above background is observed in the chloroplasts. (**B**) Another cryosection showing specific peroxisomal localization of luciferase. The plane of the section does not reveal the crystalloid structure of the peroxisome (P). Note the absence of immunolabeling in the mitochondria (m). Bar = 0.1 μm.

luciferase and thiolase (a peroxisomal enzyme of the β-oxidation pathway in *S.cerevisiae*), demonstrated that luciferase is indeed located within peroxisomes since both antibodies decorate the same organelle (Figure 2C).

We also expressed the luciferase gene in the methylotrophic yeast *Hansenula polymorpha*. The plasmid YEp13 can replicate in this yeast and the *S.cerevisiae LEU2* gene on the plasmid can complement the *H. polymorpha leu1-1* mutant (Gleeson *et al.*, 1986). Also, the alcohol oxidase gene has been expressed in *H. polymorpha* from the *S.cerevisiae* PGK promoter (Distel *et al.*, 1988). We used the plasmid pPGK-LUC-YEp134, since it is based on the plasmid YEp13, to transform the *leu1-1* strain of *H.polymorpha* to leucine prototrophy and for expression of the luciferase cDNA in this yeast species. *H.polymorpha* contains an abundance of large peroxisomes when grown on methanol as the sole carbon source [the two major methanol assimilatory enzymes, dihydroxyacetone synthase (DHAS) and alcohol oxidase (AOX), are peroxisomal proteins]. LEU^+ clones expressing luciferase were grown in methanol-containing growth medium and then processed for immunocryoelectron microscopy. Luciferase was present within the peroxisomes of this yeast, further demonstrating the ability of luciferase to be transported to peroxisomes in a wide variety of organisms (data not shown).

CAT–PMP20 fusion protein is peroxisomal in mammalian cells

The results described above demonstrate that a higher eukaryote peroxisomal protein (luciferase) can be transported

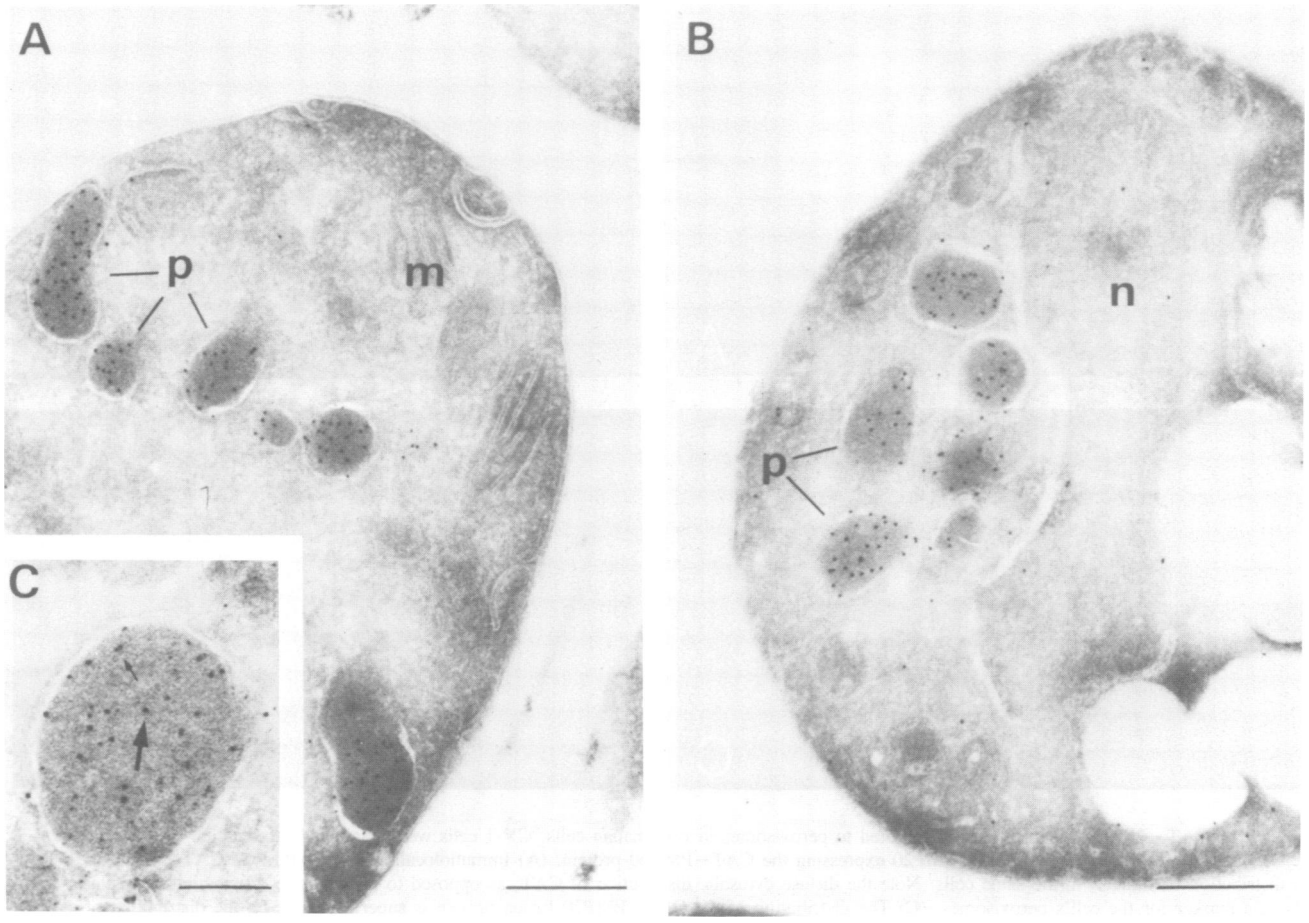

Fig. 2. Firefly luciferase is targeted to peroxisomes in *S.cerevisiae*. Immunogold labeling of oleic acid induced yeast cells expressing firefly luciferase from plasmid pPGK-LUC-YE23R. (**A**) and (**B**) Cryosections of yeast cells showing intense labeling of peroxisomes. Luciferase is only detected in the peroxisomes and not in the mitochondria (**A**) or nucleus (**B**). (**C**) Double immunolabeling of luciferase and thiolase. Antibodies against these enzymes were conjugated with protein A and gold particles of different diameter [luciferase: 10 nm gold particles, thiolase: 5 nm gold particles (arrow)]. P, peroxisome; M, mitochondria; N, nucleus. Bar = 0.1 μm.

to peroxisomes of plants and yeasts. To test whether a yeast peroxisomal protein could be transported into peroxisomes in mammalian cells, we attempted to express PMP20 (a peroxisomal protein from the yeast *Candida boidinii*; Garrard and Goodman, 1989) in CV-1 monkey kidney cells. The cDNA encoding PMP20 was placed downstream of the SV40 early promoter to create the plasmid pSV2-PMP20. This plasmid was transfected into CV-1 cells and the cells were subsequently processed for double indirect immunofluorescence in an attempt to determine the subcellular distribution of the PMP20 protein in mammalian cells. Unfortunately, we were unable to detect the protein by immunofluorescence in these experiments. A number of explanations could account for this result including a low steady-state level due to a high rate of turnover for this foreign protein; inefficient synthesis due to unusual codon composition of the gene (for mammalian cells); or inability of the monoclonal anti-PMP20 antibody (Goodman *et al.*, 1986) to recognize the protein by immunofluorescence, possibly because of an incompatible fixation procedure.

As an alternative to expression of the entire PMP20 protein in mammalian cells, we created a chimeric gene that would express a fusion protein containing a portion of the PMP20 protein appended to the cytosolic protein chloramphenicol acetyltransferase (CAT). A complementary pair of oligodeoxynucleotides that encoded the C-terminal 12 amino acids of PMP20 was hybridized and inserted at the 3′ end of the coding region of the CAT gene to create a CAT−PMP20 fusion gene. The predicted product of this gene would contain all 219 amino acids of CAT followed by the C-terminal 12 amino acids of PMP20. We chose to make the fusion protein with a short C-terminal region of PMP20 because we had previously demonstrated the existence of peroxisomal targeting information in the C-terminal regions of five other peroxisomal proteins—luciferase (Gould *et al.*, 1987, 1989), rat acyl-CoA oxidase, rat bifunctional enzyme, pig D-amino acid oxidase, and human catalase (Gould *et al.*, 1988)—and felt that the C terminus of PMP20 might contain the protein's peroxisomal targeting signal.

The plasmids pSV2CAT, encoding the wild-type CAT gene, and pSV2CAT-PMP20, containing the CAT−PMP20 fusion gene, were transfected into CV-1 cells (each independently). Two days after transfection, cells were processed for double indirect immunofluorescence using a monoclonal mouse anti-CAT antibody to detect either CAT or CAT−PMP20, and a rabbit anti-catalase antibody to localize the endogenous catalase of the cells. Since catalase is a marker for peroxisomes, the distribution of catalase marks the cell's peroxisomes. Wild-type CAT was cytosolic

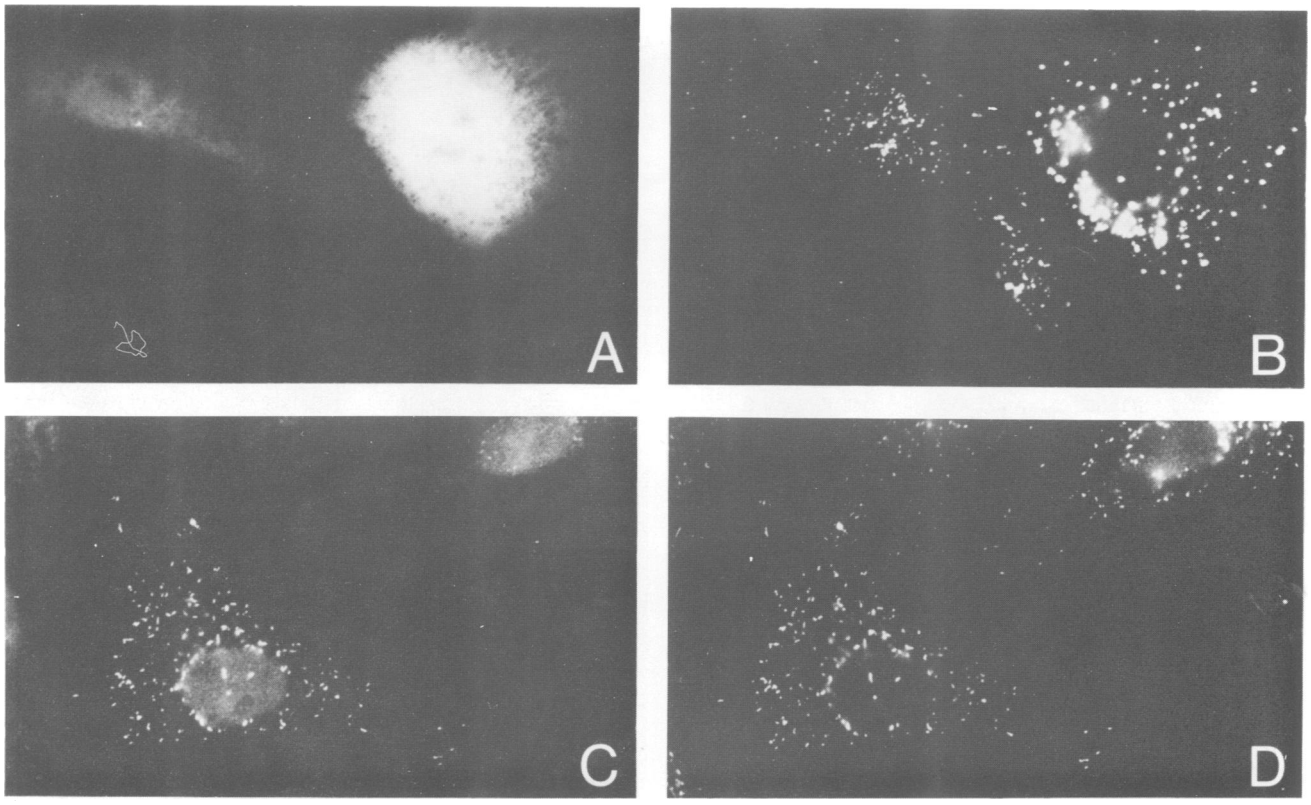

Fig. 3. The CAT−PMP20 fusion protein is targeted to peroxisomes in mammalian cells. CV-1 cells were tranfected in either pSVCAT expressing the wild-type CAT protein or pSV2CAT-PMP20 expressing the CAT−PMP20 protein. (**A**) Immunolocalization of wild-type CAT protein and (**B**) the distribution of catalase in the same cells. Note the diffuse cytosolic distribution of CAT, as opposed to the punctate distribution of catalase, which acts as a marker for the cell's peroxisomes. (**C**) The distribution of the CAT−PMP20 fusion protein is superimposable on the distribution of catalase (**D**) within the same cell, demonstrating that the fusion protein is peroxisomal. Bar = 10 μm.

(Figure 3A and B) as previously reported (Gorman *et al.*, 1982a; Gould *et al.*, 1987, 1988, 1989). In contrast, the CAT−PMP20 fusion protein was found to localize with catalase in the cell (Figure 3C and D), demonstrating that it was peroxisomal and that the yeast protein PMP20 contains a peroxisomal targeting signal within its C-terminal 12 amino acids.

Discussion

Firefly luciferase has previously been shown to be peroxisomal in insects and mammals (Keller *et al.*, 1987). We have expressed the cDNA for firefly luciferase in yeast (*S.cerevisiae* and *H.polymorpha*) and a plant (*N.tabacum*) and found that the protein is transported to peroxisomes in each of these organisms. Luciferase is also peroxisomal when expressed in cells of the *Xenopus laevis* central nervous system (C.Holt, personal communication). In addition, we have shown that the yeast peroxisomal protein PMP20 (from *C.boidinii*) contains a peroxisomal targeting signal within its C-terminal 12 amino acids that can act in mammalian cells. Thus, the signal(s) for peroxisomal protein import can be recognized in widely divergent organisms. This observation is reminiscent of the functional conservation of secretory signal sequences (Briggs and Gierasch, 1986) and mitochrondrial targeting signals (Bohni *et al.*, 1980; Scarpulla and Nye, 1986; Cheng *et al.*, 1987) between yeast and mammalian cells. Each of these findings supports the hypothesis that the mechanisms for protein import into subcellular organelles have been highly conserved during the evolutionary process.

We have recently demonstrated that the minimal peroxisomal targeting signal in luciferase consists of the C-terminal tripeptide serine-lysine-leucine (Gould *et al.*, 1989). The same report also presented data which showed that in luciferase, the serine could be replaced by either alanine or cysteine and that the lysine could be replaced by either arginine or histidine without a loss of targeting signal function. In light of these observations, the ability of the C-terminal 12 amino acis of PMP20 to function as a peroxisomal targeting signal in mammalian cells is not surprising. This protein contains a version of the minimal peroxisomal targeting signal identified in luciferase (alanine-lysine-leucine-COOH) at its C terminus and thus, a C-terminal segment of this protein would be predicted to act as a targeting signal in mammalian cells.

In addition to the reports indicating that the tripeptide serine-lysine-leucine (or conservative variants) functions as a peroxisomal targeting signal (Gould *et al.*, 1987, 1988, 1989; Miyazawa *et al.*, 1989), Small *et al.* (1988) have presented data which suggest that a yeast (*Candida tropicalis*) acyl-CoA oxidase protein is imported independently of such a signal. The possible existence of different signals for peroxisomal protein import implies that either *C.tropicalis* has evolved a unique type of peroxisomal protein import or that more than one type of signal may be used for peroxisomal protein translocation. It will be of interest to see whether the peroxisomal targeting signal identified by

Small *et al.* (1988) in *C.tropicalis* can function in other eukaryotes.

Our understanding of other protein import systems such as ER translocation and mitochondrial protein transport has been greatly advanced by genetic analysis of these processes in yeast. Several of the strategies used for the selection of transport mutants have relied on the knowledge of *cis*-acting signals that direct proteins into these compartments in yeast (Yaffe and Schatz, 1984; Deshaies and Schekman, 1987). The simplest interpretation of the import of luciferase into peroxisomes of diverse eukaryotes is that the same peroxisomal targeting signal is being recognized in each species. If so, the tripeptide serine-lysine-leucine (or a conservative variant) should be sufficient to direct peroxisomal protein import in plants, insects and yeast, as well as in mammalian cells. Experiments to test this possibility are currently under way. Identification of a peroxisomal targeting signal in yeast may lead to strategies for a genetic analysis of peroxisomal protein import in this organism.

Materials and methods

Reagents

The *H.polymorpha* strain *leu1-1* (Gleeson *et al.*, 1986) was supplied by M.Gleeson (Sibia, La Jolla, CA) and the *S.cerevisiae* strain BJ1991 has been described previously (Distel *et al.*, 1987). Anti-thiolase antibodies were a gift from W.H.Kunau (University of Bochum, FRG) and the anti-CAT producing hybridoma line CAT-2 was gift from C.Gorman (Genentech, San Francisco, CA). The rabbit anti-catalase antibody was a gift from A.Schram (University of Amsterdam, Amsterdam, The Netherlands). Rabbit and guinea-pig antibodies raised against luciferase have been described earlier (Keller *et al.*, 1987). Yeast media were prepared as described elsewhere (Gleeson *et al.*, 1986; Veenhuis *et al.*, 1987). All other reagents were obtained from standard sources.

Plasmids

All manipulations of DNA were performed essentially as described by Maniatis *et al.* (1982). The structures of plasmids used for the generation of the luciferase-expressing transgenic plants have been described earlier (Ow *et al.*, 1986). pMA91-LUC was created by cleaving pJD201 (de Wet *et al.*, 1987) with *Bsm*I, making the ends flush with Klenow fragment of *E.coli* DNA polymerase I, ligating *Bgl*II linkers onto the ends, digesting with *Bgl*II and *Bam*HI and ligating the 1850 bp fragment containing luciferase into the *Bgl*II site of pPGK between the 5′ and 3′ flanking regions of the *S.cerevisiae* PGK gene and screening recombinant clones for inserts with the correct orientation. pPGK consists of the *Hin*dIII fragment of pMA91 (a gift from Dr S.M.Kingsman) containing both the 5′ and 3′ flanking regions of the PGK gene inserted in the *Hin*dIII site of pBR322. pPGK-LUC was digested with *Hin*dIII (sites for which border the entire PGK−LUC transcription unit) and the 3.5 kb fragment containing the PGK promoter and the luciferase cDNA was inserted between the *Hin*dIII sites of YEp13 and YE23R (Distel *et al.*, 1987) to generate pPGK-LUC-YEp13 and pPGK-LUC-YE23R respectively.

pSV2CAT has been described earlier (Gorman *et al.*, 1982b), as has pCATC (Gould *et al.*, 1988). Two oligodeoxynucleotides with the sequences 5′-AGCTTGTCGACGTATCTACTGCCCAAAAGATTATTGCCAAA-TC-3′ and 5′-CTAGGATTTGGCAATAATCTTTTGGGCAGTAGATA-CGTCGACA-3′ were hybridized to each other to create a double-stranded segment of DNA with 4 nt long, single-stranded overhangs compatible with those generated by *Hin*dIII and *Xba*I. This DNA molecule was ligated between the *Hin*dII and *Xba*I sites of pUC19 to create pUC-PMP20. The short insert could potentially encode the C-terminal 12 amino acids of PMP20 and contained a cleavage site of *Hinc*II at the 5′ end of the 5′-most codon in this sequence. To form pCAT-PMP20, pUC-PMP20 was cleaved with *Hinc*II and *Ssp*I and the resultant 450 bp fragment was isolated and ligated between the *Nae*I and *Ssp*I sites of pCATC, creating a gene fusion in which all 219 codons of the CAT gene were followed by the 3′-most 12 codons of the PMP20 cDNA and a stop codon. The 700 bp *Hin*dIII−*Bam*HI fragment containing this fusion gene was cleaved from pCAT-PMP20 and inserted between the *Hin*dIII and *Bgl*II sites of pSV2dhfr (Subramani *et al.*, 1981) to create pSV2CATC-PMP20. This plasmid contained the CAT−PMP20 fusion gene under the transcriptional control of the SV40 early promoter.

Plant, yeast and mammalian cell transfections

Transgenic tobacco (*N.tabacum*) plants expressing luciferase were produced using *Agrobacterium* Ti-plasmid transformation as described by Ow *et al.* (1986). The luciferase cDNA was linked to the cauliflower mosaic virus 35S RNA promoter and the nos 3′ region (represented by the construct pD0432. Ow *et al.*, 1986) and inserted into an *Agrobacterium* Ti-plasmid.

Yeast strains were grown in YEPD medium under control conditions, in SD-ura for selection and maintenance of the pPGK-LUC-YE23R *S.cerevisiae* transformants and in SD-leu for selection and maintenance of the pPGK-LUC-YEp13 *H.polymorpha* transformants. To create the yeast strains expressing luciferase, the plasmid pPGK-LUVC-YE23R was used to transform the *S.cerevisiae* strain BJ1991 (α, *leu2, trpl, ura3-52, prbl-1122, pep4-3*) (Jones, 1977) to *URA3*$^+$ by the method of Itoh *et al.* (1983). pPGK-LUC-YEp13 was used for transformation of the *H.polymorpha* strain *leu1-1* to leucine prototrophy by the method of Gleeson *et al.* (1986). For electron microscopy experiments, the *S.cerevisiae* strains were grown under conditions that provide for induction of peroxisomes (Veenhuis *et al.*, 1987); the *H.polymorpha* cells were switched from SD-leu to synthetic medium lacking leucine supplemented with 0.5% methanol (Gleeson *et al.*, 1986) and grown to mid-log phase before fixation.

CV-1 monkey kidney cells were grown on glass coverslips as described (Keller *et al.*, 1987) and transfected using the calcium phosphate precipitate technique described by Parker and Stark (1979).

Immunocryoelectron microscopy

Leaf protoplasts from luciferase-expressing transgenic tobacco plants were prepared as described (Maligna, 1984) and fixed with 0.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) and 7.5% sucrose for 1 h. Ultrathin frozen sections were prepared for cryoultramicrotomy as described by Tokuyasu (1980). Immunolabeling and embedding of the immunolabeled frozen sections in acrylic resin LR-white (London Resin) have been described elsewhere (Keller *et al.*, 1984). The sections were observed without post-staining in a Philips model 300 transmission electron microscope equipped with an 11 μm diameter aperture at a tension of 80 kV.

Yeast cells were fixed with 2% paraformaldehyde, 0.5% glutaraldehyde and 0.1 M sodium phosphate buffer (pH 7.4) for 2 h at room temperature. Subsequently cells were washed with sodium phosphate buffer, treated with 1% sodium periodate for 1 h, washed and resuspended in 1% ammonium chloride for 30 min to block free aldehyde groups. After two washes with buffer, cells were imbedded in 10% gelatin. Samples were post-fixed in fixative solution for 1 h on ice, washed and stored in a 2% paraformaldehyde solution in 0.1 M sodium phosphate buffer. Before sectioning, samples were infused with a 2.3 M sucrose solution in 0.1 M sodium phosphate and frozen in liquid nitrogen. Ultrathin frozen sections were prepared in a Reichert-Jung Fc4 cryo-ultra microtome. The thawed sections were mounted on carbon-coated grids and collected on 2% gelatin plates. After heating of the gelatin plates, sucrose was allowed to diffuse out of the sections for 10 min at room temperature. Immunolabeling of the sections was carried out essentially as described by Slot and Geuzes (1981). Immunolabeled sections were post-stained and imbedded in a 0.3% uranyl acetate−1.5% methyl cellulose mixture and examined in the electron microscope.

Immunofluorescence microscopy

Immunofluorescence was performed essentially as described earlier (Keller *et al.*, 1987). CV-1 monkey kidney cells were transfected with either pSV2CAT, pSV2CATC-PMP20. Forty-eight hours after transfection, the cells were washed twice with 5 ml phosphate-buffered saline (PBS, pH 7.2), fixed in 3% formaldehyde/PBS for 10−30 min and permeabilized with a solution of 1% Triton X-100/PBS for 5 min. Cells transfected with either pSV2CAT or pSV2CATC-PMP20 alone were then incubated with a rabbit anti-catalase antibody and the CAT-2 monoclonal anti-CAT antibody followed by a fluorescein conjugate of a goat anti-mouse IgG antibody and a rhodamine conjugate of a goat anti-rabbit IgG antibody.

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