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A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase

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Several peroxisomal proteins do not contain the previously identified tripeptide peroxisomal targeting signal (PTS) at their carboxy-termini. One such protein is the peroxisomal 3-ketoacyl CoA thiolase, of which two types exist in rat [Hijikata *et al.* (1990) *J. Biol. Chem.*, 265, 4600-4606]. Both rat peroxisomal thiolases are synthesized as larger precursors with an amino-terminal prepiece of either 36 (type A) or 26 (type B) amino acids, that is cleaved upon translocation of the enzyme into the peroxisome. The prepieces are necessary for import of the thiolases into peroxisomes because expression of an altered cDNA encoding only the mature thiolase, which lacks any prepiece, results in synthesis of a cytosolic enzyme. When appended to an otherwise cytosolic passenger protein, the bacterial chloramphenicol acetyltransferase (CAT), the prepieces direct the fusion proteins into peroxisomes, demonstrating that they encode sufficient information to act as peroxisomal targeting signals. Deletion analysis of the thiolase B prepiece shows that the first 11 amino acids are sufficient for peroxisomal targeting. We conclude that we have identified a novel PTS that functions at amino-terminal or internal locations and is distinct from the C-terminal PTS. These results imply the existence of two different routes for targeting proteins into the peroxisomal matrix.
Key words: 3-ketoacyl-CoA thiolase/peroxisome/peroxisome biogenesis/peroxisomal targeting signal/topogenesis.

Introduction

Signals that target proteins into the ER, mitochondria or chloroplasts are generally found in amino-terminal peptides that are removed upon entry of the proteins into the organelles (reviewed by Verner and Schatz, 1988). This contrasts with the targeting of proteins into microbodies, a group of organelles which include peroxisomes, glyoxysomes and glycosomes. Most microbody proteins are not proteolytically processed (see Borst, 1986) and several peroxisomal proteins have been shown to contain a peroxisomal targeting signal (PTS) at their carboxy-termini (Gould *et al.*, 1987, 1988; Miyazawa *et al.*, 1989). The carboxy-terminal PTS consists of a tripeptide with the consensus sequence: ser or ala or cys-lys or arg or his-leu (Gould *et al.*, 1989). This PTS is present in many peroxisomal, glyoxysomal and glycosomal proteins (Gould *et al.*, 1990b; Keller *et al.*, 1991) and its function has been conserved during eukaryotic evolution (Gould *et al.*, 1990a; Fung and Clayton, 1991). Keller *et al.* (1991) have therefore proposed to rename this particular PTS as a microbody targeting signal (McTS).

The amino acid sequences of several microbody proteins do not contain the conserved tripeptide McTS at their carboxy-termini (see Gould *et al.*, 1989). One such protein is the peroxisomal 3-ketoacyl-CoA thiolase (E.C. 2.3.1.16; referred to as thiolase herein), that catalyzes the last step of the fatty acid β-oxidation cycle. Fatty acid β-oxidation systems in mammals are found both in mitochondria and in peroxisomes. Three distinct molecular species of thiolase have been identified in rat by the analysis of their cDNA or gene sequences: one mitochondrial thiolase (Arakawa *et al.*, 1987) and two peroxisomal isoenzymes, thiolase A and B (or 2 and 1), respectively (Hijikata *et al.*, 1987, 1990; Bodnar and Rachubinski, 1990). The peroxisomal thiolase A is constitutively expressed whereas the expression of thiolase B is strongly induced by peroxisome proliferators such as hypolipidemic drugs (Bodnar and Rachubinski, 1990; Hijikata *et al.*, 1990). The peroxisomal thiolases A and B are unusual peroxisomal proteins in that they are synthesized as larger precursors with amino-terminal prepieces of 36 and 26 amino acids respectively, that are cleaved upon translocation of the protein into peroxisomes (Hijikata *et al.*, 1990; Bodnar and Rachubinski, 1990). Except for the extra 10 amino acids at the amino-terminus of the thiolase A prepiece and some 10 amino acid substitutions in the rest of the molecules, both peroxisomal thiolases are identical. It is not clear whether proteolytic processing of the prepiece is directly coupled to import into peroxisomes. Pulse-labeling of thiolase, followed by subcellular fractionation provided no evidence for import-dependent processing of the enzyme (Miura *et al.*, 1984). On the other hand, in human fibroblasts from patients with Zellweger's syndrome (a condition characterized by defects in peroxisome biogenesis) only the larger, precursor form of thiolase was detected, suggesting that the proteolytic processing does depend upon a functional peroxisomal import system (Schram *et al.*, 1986). We analyzed the role of these prepieces in targeting thiolase into the peroxisome. Our results show the presence of a novel PTS in the prepieces of the peroxisomal thiolases, different from the previously described carboxy-terminal tripeptide McTS.

Results

The rat thiolase prepiece is both necessary and sufficient for targeting proteins into peroxisomes

In order to study both thiolase isoenzymes, we used a cDNA clone for the peroxisomal thiolase A to create an artificial

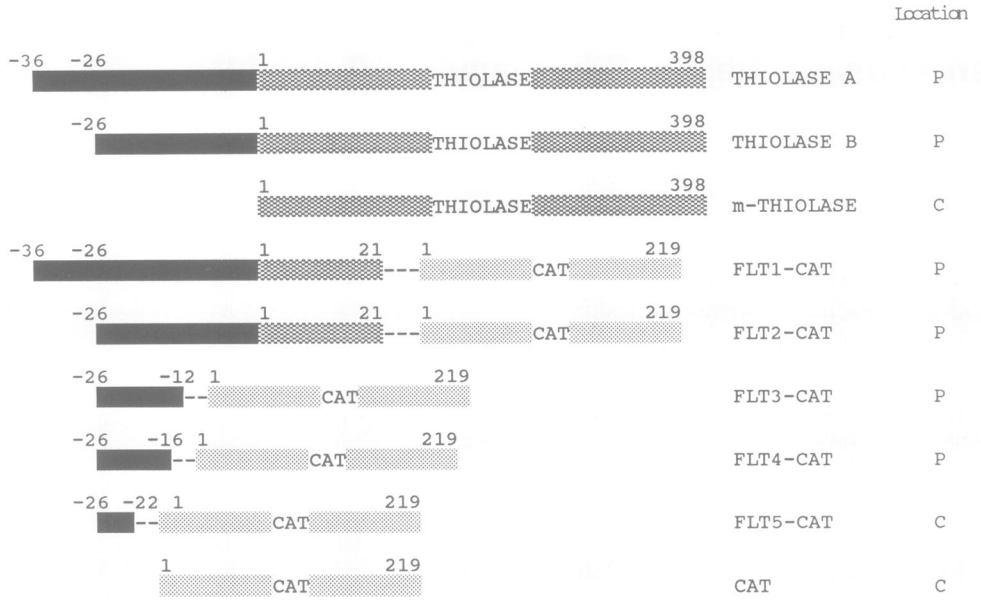

Fig. 1. Structures of the thiolase and thiolase–CAT fusion genes. Black bars represent the thiolase prepieces, shaded bars represent mature thiolase or CAT sequences as indicated. Lengths of the bars are not to scale. The numbers above each construct denote amino acid numbers. The negative numbers refer to the thiolase prepieces. The subcellular locations of the gene products, as determined by immunofluorescence, are indicated (P = peroxisomal, C = cytosolic).

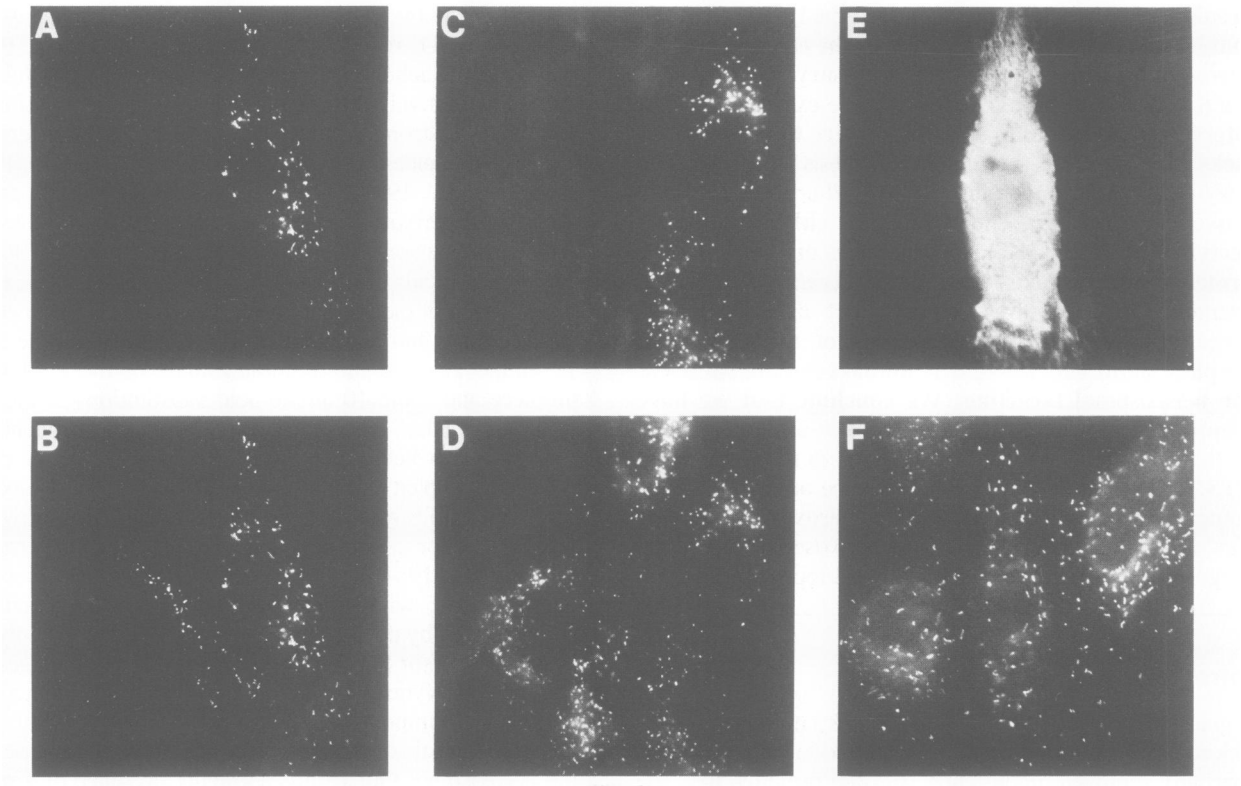

Fig. 2. Immunofluorescent localization of thiolase A, thiolase B and mature thiolase. CV-H Px110 cells were transfected either with pRSV–FLT1–thiol, pRSV–FLT2–thiol or pRSV–thiol-40, encoding the rat thiolase A, B and mature thiolase, respectively. Cells were processed for double indirect immunofluorescence using an anti-CAT mouse monoclonal antibody to detect the peroxisomal CAT–PMP20 fusion protein expressed by the CV-H Px110 cells and an anti-rat thiolase rabbit antibody to detect the thiolase proteins. Secondary antibodies used were a rhodamine conjugate of a goat anti-mouse Ig antibody and a fluorescein conjugate of a goat anti-rabbit Ig antibody. The upper micrographs show the distribution of (**A**) thiolase A, (**C**) thiolase B and (**E**) mature thiolase. The locations of peroxisomes in these cells is shown in the lower micrographs, (**B**), (**D**) and (**F**), respectively.

thiolase B cDNA. To this end we deleted the initiation codon of the thiolase A cDNA. This truncated thiolase A cDNA will direct the initiation of protein synthesis from the second methionine codon in thiolase A cDNA, which corresponds to the initiation codon of the thiolase B cDNA (see Figure 1). Plasmids directing the expression of these sequences from

A.

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-36                 -26          -20                     -10              -1  1              10
M S E S V G R T S A M - H R L Q V V L G H L - A G R P - - E S S S A L Q A A P C↓ S A T F P Q A S A S D V V V V   Rat A
                    M - H R L Q V V L G H L - A G R S - - E S S S A L Q A A P C↓ S A G F P Q A S A S D V V V V   Rat B
                    M - Q R L Q V V L G H L - R G P A - - D S G W M P Q A A P C↓ L S G A P Q A S A A D V V V V   Human
                    M S Q R L Q S I K D H L - - - V L S A M G K G E S K R K N S L L - - - - E K R P E D V V I V   S.cerevisiae
                    M - D R L N Q L S G Q L - - - - - - - - - - - - K P N A K Q S I L - - - - Q K N P D D V V I V   C.tropicalis
                    M - D R L N N L A T Q L - - - - - - - - E Q N P A K G L D A I T - - - - S K N P D D V V I T   Y.lipolytica
          M Q P I P D V N Q R I A R I S A H L H P P K S Q M E E S S A L R R A N C R↓ ——malate dehydrogenase——   gMDH
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B.

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-26         -20              -12                                              Location
M - H R L Q V V L G H L - A G R S s g t   CAT   FLT3-CAT      peroxisomal
M - H R L Q V V L G H L - - - - s s g t   CAT   FLT4-CAT      peroxisomal
M - H R L Q - - - - - - - - - - s s g t   CAT   FLT5-CAT        cytosolic
1         5           11        15
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Fig. 3. (**A**) Amino acid sequence comparison of the amino-termini of mammalian and yeast peroxisomal thiolases and a plant glyoxysomal malate dehydrogenase. The numbering refers to the rat peroxisomal thiolase A. Dashes indicate deletions necessary to align the sequences. Amino acids that are identical are shown in bold type. The arrow indicates the cleavage site between the leader and the mature enzyme. Sequences are: rat peroxisomal thiolase A and B, respectively (Hijikata *et al.*, 1990); human peroxisomal thiolase (Bout *et al.*, 1988); *Saccharomyces cerevisiae* peroxisomal thiolase (B.von Geldern and W.-H.Kunau, personal communication); *Candida tropicalis* peroxisomal thiolase (U.Stank, T.Kamiryo and W.-H.Kunau, personal communication); *Yarrowia lipolytica* peroxisomal thiolase (G.Berninger and E.Schweizer, personal communication); water melon glyoxysomal malate dehydrogenase (Gietl, 1990). (**B**) The amino-terminal sequences of FLT3−CAT, FLT4−CAT, and FLT5−CAT are shown. Their subcellular localization is described on the right.

the Rous sarcoma virus long terminal repeat were introduced into a cell line (CVH Px110; derived from CV-1 monkey kidney cells) by calcium phosphate mediated transfection. The CVH Px110 cell-line has been engineered to express the artificial peroxisomal protein CAT−PMP20 (Gould *et al.*, 1990a), which can be detected by indirect immunofluorescence using a primary mouse monoclonal anti-CAT antibody. The cells were subsequently processed for double immunofluorescence to determine the subcellular distribution of the transiently expressed proteins. Figure 2B shows the punctate fluorescence, characteristic of peroxisomes, in two CAT−PMP20 expressing cells, one of which also expressed thiolase A (Figure 2A). It is clear that thiolase A co-localized with CAT−PMP20 in the peroxisomes. Note that no thiolase was detected in the other cell in the panel. This is probably due to the low expression level of the endogenous thiolase in CV-1 or CVH Px110 cells, not allowing detection by immunofluorescence. Figure 2C and D show that similar results were obtained with thiolase B. Under these conditions we did not find any difference between the two forms of thiolase that might provide clues as to the function of the extra 10 amino acids on the thiolase A prepiece.

Using PCR we constructed a thiolase gene in which protein synthesis initiates at a position corresponding to the first amino acid of the mature thiolase (Figure 1). Figure 2E and F show that when expressed in CV-1 cells, mature thiolase was no longer peroxisomal but cytosolic. This demonstrates that the thiolase prepieces are required for import of the enzymes into peroxisomes.

To examine whether the thiolase prepieces were also sufficient for import into peroxisomes, we fused DNAs encoding the thiolase A and B prepieces in-frame to the 5′ end of the coding region of the CAT gene. Since we did not know whether proteolysis was necessary for import of thiolase, the 21 amino-terminal amino acids of mature thiolase were also included in this fusion (Figure 1). We used the anti-CAT mouse monoclonal antibody to detect the expression of these fusion proteins in CV-1 cells and a rabbit anti-SKL antibody to label their peroxisomes (Gould *et al.*, 1990b). We observed a weak but punctate fluorescence using the anti-CAT antibody to label the FLT1−CAT and FLT2−CAT fusion proteins, respectively (data not shown). In both cases the weak fluorescence was superimposable with the fluorescence obtained when using the anti-SKL antibodies. We conclude that the thiolase sequences present in these fusion proteins are sufficient to direct the import of the otherwise cytosolic CAT (Gorman *et al.*, 1982a; Gould *et al.*, 1987, 1988, 1989) into peroxisomes even though these fusion proteins are not efficiently expressed and/or detected. We had no problems in detecting CAT when much shorter sequences were fused onto the CAT amino-terminus (see below). One explanation for the poor detection of the FLT1− and FLT2−CAT fusion proteins could be that the extra sequences on the amino-terminus of CAT interfere with recognition of the fusion proteins by the anti-CAT monoclonal antibody, either by direct steric interference or by partial disruption of a conformational epitope on the CAT protein.

Deletion analysis of the thiolase prepiece

Figure 3A shows a sequence comparison of the prepieces of both rat thiolases with the amino-termini of several peroxisomal thiolases from other species and the prepiece of the watermelon glyoxysomal malate dehydrogenase (gMDH). Significant positional identity was found near the amino-termini of these sequences, whereas the carboxy-terminal parts of both rat prepieces showed similarity with those of the human thiolase and gMDH sequences, both of which are also cleaved at similar positions. It is therefore reasonable to assume that the amino-terminal part of the thiolase prepieces might be required for the peroxisomal targeting and that the carboxy-terminal part might be involved in the proteolytic processing of the prepieces.

To focus on the targeting of thiolase, we constructed genes encoding the first 15, 11 or 5 amino acids of the thiolase B prepiece fused through a small linker onto the amino-terminus of CAT (see Figure 3B). It is clear from Figure 4A, B and C, and D that the first 15 or 11 amino

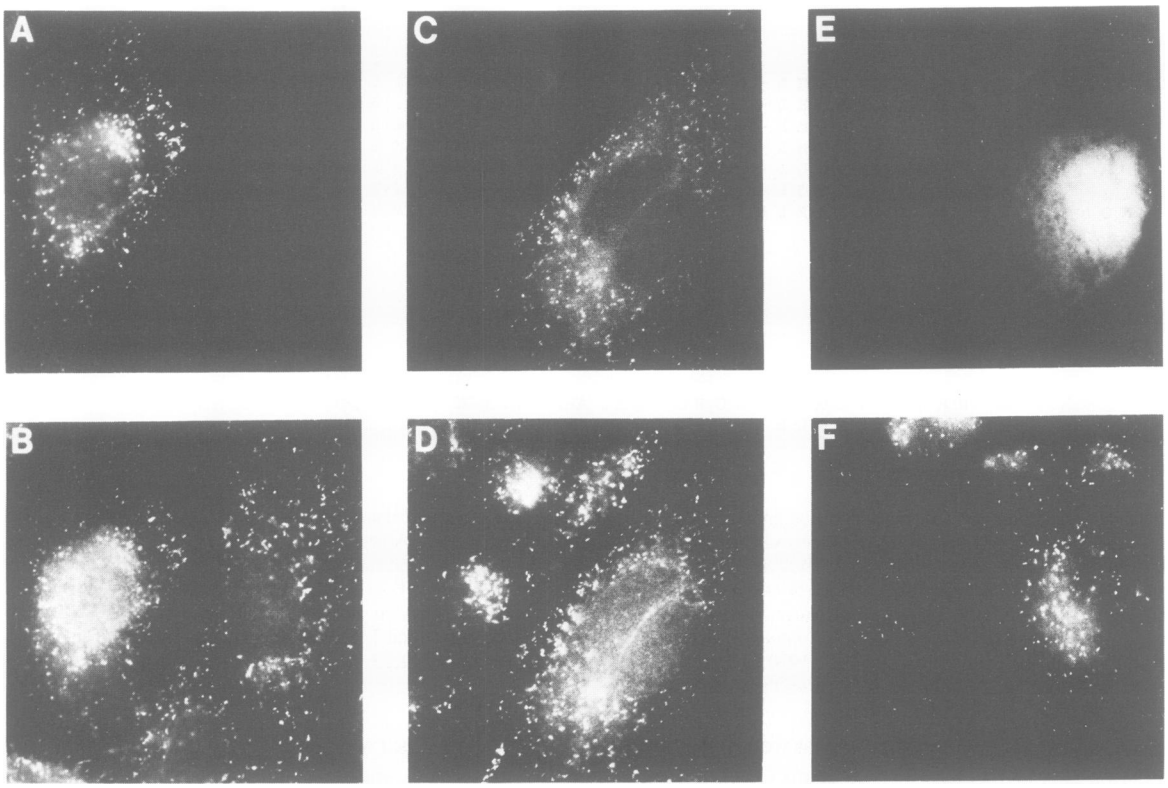

Fig. 4. Immunofluorescent localization of thiolase−CAT deletion mutants. CV-1 cells were transfected either with pRSV−FLT3−CAT, pRSV−FLT4−CAT or pRSV−FLT5−CAT (see Figure 3B). Cells were processed for double indirect immunofluorescence using an anti-CAT mouse monoclonal antibody to detect the thiolase−CAT fusion proteins and an anti-SKL rabbit antibody to label peroxisomes (see Gould *et al.*, 1990b). Secondary antibodies used were a fluorescein conjugate of a goat anti-mouse Ig antibody and a rhodamine conjugate of a goat anti-rabbit Ig antibody. The upper micrographs show the distribution of (**A**) FLT3−CAT, (**C**) FLT4−CAT and (**E**) FLT5−CAT, respectively. The locations of peroxisomes in these cells is shown in the lower micrographs, (**B**), (**D**) and (**F**), respectively.

acids of the thiolase prepiece, respectively, were sufficient to target CAT into the peroxisomes. However, the peroxisomal targeting by the 11 amino acid prepiece seemed less efficient than that of the 15 amino acid prepiece, as evidenced by some cytosolic fluorescence in many FLT4−CAT expressing cells (compare Figure 4A with C). Though a qualitative result, we observed this phenomenon in several independent experiments. The lack of punctate fluorescence in Figure 4E shows that the five amino acid prepiece was no longer able to target CAT to peroxisomes. We therefore conclude that the first 11 amino acids of the thiolase B prepiece either constitute or contain a novel PTS.

As a control to ensure that the thiolase−CAT fusion proteins were actually translocated into the peroxisomes and not simply aggregated at the outer periphery of the peroxisome, we examined sets of transfected cells permeabilized with the detergent digitonin. Whereas 1% Triton X-100 permeabilizes both the plasma membrane and the peroxisomal membrane, a low concentration (25 μg/ml) digitonin solution will permeabilize only the plasma membrane, not the peroxisomal membrane (Wolvetang *et al.*, 1990). Thus, while peroxisomal proteins cannot be detected in immunofluorescence experiments in which the cells are permeabilized with 25 μg/ml digitonin, cytosolic proteins, or proteins exposed to the cytosol, can be detected under these conditions. In cells transfected with the plasmid pSV2CAT, CAT protein (a cytosolic protein) was as easily detected in cells permeabilized with digitonin (Figure 5B) as it was in cells permeabilized with Triton X-100 (Gould *et al.*, 1987; 1988; 1989). However, in cells transfected with pRSV−FLT3−CAT, the peroxisomal staining observed for the FLT3−CAT fusion protein was detected only after permeabilization of the cells with Triton X-100 (Figure 4A,B) and never after permeabilization with digitonin alone. In digitonin-permeabilized cells (transfected with pRSV−FLT3−CAT), the FLT3−CAT protein was only detected in the cytosol of the few cells that expressed such a large amount of the fusion protein that not all could be imported into peroxisomes, leaving some in the cytosol (Figure 5A). The inability of the digitonin treatment to permeabilize peroxisomes was confirmed by the fact that the endogenous peroxisomal catalase of CV-1 cells could not be detected in these cells (Figure 5C). Digitonin has no general effect on the specificity or affinity of the antibodies we used since normal peroxisomal staining of catalase and FLT3−CAT was observed in cells permeabilized with 25 μg/ml digitonin plus Triton X-100 (data not shown). Taken together, these results demonstrate that the thiolase−CAT fusion protein FLT3−CAT, and likely the others, are translocated into the peroxisome and are not concentrated on the outer surface of the organelle where they would be accessible to the cytosol.

Import of the peroxisomal thiolases is associated and/or followed by a proteolytic processing event which removes the prepieces from the proteins. In CV-1 cells, which normally express very little peroxisomal thiolase, Western blotting experiments revealed that neither the thiolase A (data not shown) nor B were appreciably processed (Figure 6).

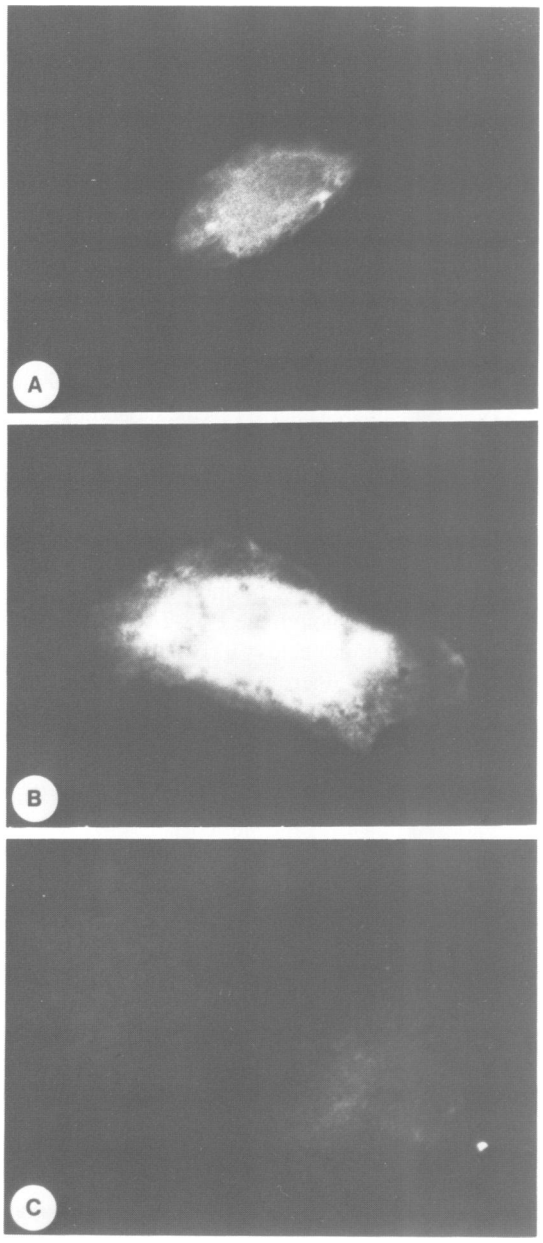

Fig. 5. Peroxisomal FLT3−CAT cannot be detected in digitonin-permeabilized cells. CV-1 cells were transfected with either pRSV−FLT3−CAT or pSV2CAT and processed for immunofluorescence microscopy using the anti-CAT antibody (A and B) and the anti-catalase antibody (C) as before with the exception that the cells were permeabilized with 25 μg/ml digitonin in PBS instead of 1% Triton X-100 in PBS. The micrographs show (**A**) a rare pRSV−FLT3−CAT transfected cell expressing such a large amount of FLT3−CAT that detectable quantities can be visualized in the cytosol after digitonin permeabilization (note the complete lack of punctate fluorescence; (**B**) cytosolic CAT protein in a cell transfected with pSV2CAT; (**C**) CV-1 cells permeabilized with digitonin and processed for immunofluorescence using antibodies to catalase; only background staining can be detected.

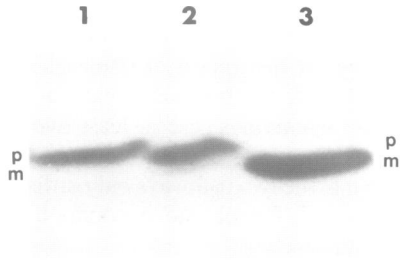

Fig. 6. Western blot of thiolase B, FLT3−thiolase, and mature thiolase expressed from plasmids in CV-1 cells. Cells were transfected with pRSV−FLT2−thiol, pRSV−FLT3−thiol, and pRSV−thiol-40, lysed in sample buffer and used for Western blot analysis with rabbit anti-thiolase antibody as described in Materials and methods. An autoradiograph of the filter is shown. Lane 1, thiolase B; lane 2, FLT3−thiolase; lane 3, mature thiolase. Thiolase B contains the proteolytic cleavage site in the leader and yet is not cleaved. FLT3−thiolase lacks the presumptive cleavage site. The precursor (p) and mature (m) forms of thiolase B are shown.

Discussion

We have identified a PTS in the cleavable prepieces of the rat peroxisomal thiolases A and B. The thiolase PTS differs from the previously described carboxy-terminal tripeptide McTS (Gould *et al.*, 1989) in several respects: the thiolase PTS is found near the amino-terminus of the polypeptide; it is cleaved from the enzyme upon maturation in the peroxisome; and it does not contain a tripeptide that conforms to the SKL-type McTS consensus sequence. We therefore conclude that we have identified a novel PTS.

Most importantly, our results imply the existence of at least two pathways for targeting proteins into the peroxisomal matrix, each involving one of the two PTSs, the carboxy-terminal tripeptide McTS on the one hand and the thiolase PTS (PTS-2) on the other hand. Additional evidence for the existence of these two pathways comes from the study of several inherited human disorders in which either peroxisomal structure or metabolic functions are defective (for reviews, see Moser, 1986; Schutgens *et al.*, 1986). In one of these diseases (i.e. Zellweger's syndrome) peroxisomes are not morphologically detectable (Goldfischer *et al.*, 1973) and peroxisomal enzymes seem to be present in the cytosol where they either accumulate or are unstable (Schram *et al.*, 1986). Santos *et al.* (1988a,b) found that in cells from Zellweger's syndrome patients membrane vesicles are present, referred to as peroxisome 'ghosts', containing peroxisomal integral membrane proteins but lacking peroxisomal matrix proteins such as catalase. Consequently, these vesicles sediment at lower densities than normal peroxisomes. Recently, thiolase was found to be associated with these peroxisome 'ghosts' in its unprocessed precursor form (Balfe *et al.*, 1990). Protease protection experiments indicate that the thiolase precursor is present inside the 'ghosts'. These data are consistent with our finding that thiolase is imported into peroxisomes by a PTS-2 dependent pathway different from the McTS pathway used by most of the peroxisomal matrix proteins (Gould *et al.*, 1989). It is interesting to speculate that the lack of processing of thiolase in 'ghosts' is due to the possibility that the processing protease may be imported by the McTS pathway. There is also evidence for these two peroxisomal import pathways in yeast. Several mutants of *S.cerevisiae* that are impaired in peroxisome assembly have been isolated (Erdmann *et al.*, 1989). In one of these mutants, of all tested peroxisomal enzymes only thiolase was not imported into peroxisomes and found to be soluble in the cytosol (M.Marzioch and W.-H.Kunau, personal communication). This mutant cannot be complemented with a wild-type thiolase gene, excluding a *cis*-mutation in the thiolase PTS.

This means that a gene specific for the import of, thus far only, thiolase is affected, and therefore demonstrates a separate pathway for the import of thiolase, also in yeast. We therefore conclude that both yeast and mammalian cells, and probably all eukaryotes, use at least two independent pathways to import proteins into peroxisomes. Of course we cannot exclude that the two pathways only differ in the initial (receptor) steps and funnel into a common downstream (translocation) machinery.

A comparison of the first 11 amino acids of the thiolase B prepiece with the corresponding amino acid sequences of peroxisomal thiolases from several other organisms and the watermelon glyoxysomal malate dehydrogenase (gMDH) reveals several conserved elements that could be required for PTS-2 function (Figure 3A). First, the R-L-Q tripeptide in positions −24 to −22, which we initially thought could be a novel tripeptide PTS analogous to the McTS. However, this is ruled out by the cytosolic location of the FLT5−CAT fusion protein. Second, there is an aliphatic residue in position −19 in all sequences. Finally, at the carboxy-terminus of the 11 amino acid sequence a conserved H-L dipeptide is found (Q-L in the *Candida tropicalis* and *Yarrowia lipolytica* sequences). Our results thus far indicate that at most 11 amino acids are required for PTS-2 function. Further analysis may show that the minimal PTS-2 is even smaller. However, as both the arginine at position −24 and the leucine at position −17 are invariably present in all sequences, they are likely to be important for PTS-2 function and may mark the boundaries of the minimal PTS-2: nine amino acids (not counting the initiator methionine). Gietl (1990) speculated that the A-H-L tripeptide in the gMDH prepiece, which conforms to the McTS consensus sequence and is present as a G-H-L in the thiolase prepieces, might be the targeting signal. We do not agree for several reasons. First, despite numerous speculations, the McTS has never been shown to function at internal locations. In fact, the addition of two or even a single amino acid onto the carboxy-terminal leucine of the McTS has been shown to abolish its function (Gould *et al.*, 1989). Second, the three yeast thiolase sequences show non-conservative substitutions in this tripeptide (Figure 3A). Third, if the McTS can indeed function at internal locations, it is unclear why the numerous non-peroxisomal proteins containing the signal randomly at internal locations are excluded from peroxisomes. Most importantly, however, the evidence for the existence of two independent peroxisomal targeting pathways presented above excludes a McTS related signal in thiolase and probably also in gMDH.

In thiolase A as well as in gMDH, the PTS-2 is not situated at the extreme amino-terminus of the prepiece but is preceded by 10 or 7 amino acids, respectively. We have found that both thiolases A and B are processed inefficiently or not at all when expressed in CV-1 cells as judged by Western blotting. Nonetheless, immunofluorescence of these same cells shows that in each case most of the protein is imported into peroxisomes. The import of these proteins into peroxisomes in the absence of significant proteolytic processing is not surprising because cells from Zellweger's syndrome patients also accumulate unprocessed thiolase in the peroxisome 'ghosts'. Despite the presence of the extra 10 amino acids on the thiolase A amino-terminus, the PTS-2 is functional and can therefore be considered an internal targeting signal in the sense that it does not need to be placed at the extreme amino-terminus of the protein. Whether it can function at other internal locations remains to be tested.

We have not found, thus far, a PTS-2 related sequence in any protein other than those listed in Figure 3A. However, as we do not yet know the functional sequence requirements of the PTS-2, it is likely that we would have missed any PTS-2 containing proteins if present in the databases we searched.

The amino-terminal location and the cleavage of the PTS-2 sequence in the thiolase leader raise obvious questions regarding the mechanism by which the PTS-2 sequence is distinguished from other amino-terminal targeting signals such as those involved in ER, mitochondrial or chloroplast targeting? Comparisons of numerous secretory signal peptides (von Heijne, 1990) have revealed that they typically possess three distinct domains: an amino-terminal positively charged region (n-region, 1−5 residues long); a central hydrophobic part (h-region, 7−15 residues); and a more polar carboxy-terminal domain (c-region, 3−7 residues). Statistical data suggest that Pro residues are generally not tolerated in the −2, −3 or +1 positions relative to the signal peptide cleavage site. Though the PTS-2 sequence contains positively charged amino acids at positions −24 and −25 in the two rat thiolases, there is no significant hydrophobic stretch between this region and the sites at which the thiolase leaders are cleaved to generate the mature thiolase (Figure 3). Furthermore, the presence of a Pro at position −2 relative to the cleavage site in PTS-2 suggests that the sequence around this cleavage site is also unlike that found in ER signal sequences. Mitochondrial targeting sequences contain positively charged amino acids, are rich in hydroxylated amino acids, can form amphipathic helices and often contain Arg residues at positions −2 or −10 relative to the cleavage site (von Heijne *et al.*, 1989). The PTS-2 sequence (positions −26 to −16) completely lacks hydroxylated amino acids and does not exhibit a tendency to form a amphipathic helix. There is also no Arg residue at positions −2 or −10 relative to the cleavage sites in the thiolase leaders. The PTS-2 sequence is also distinct from chloroplast targeting signals which are enriched in Ser and Thr residues and possess a semi-conserved sequence around the cleavage site for the stromal protease (von Heijne and Nishikawa, 1991). Thus the PTS-2 sequence appears to be distinct from ER, mitochondrial and chloroplast targeting signals and is consistent with its role in the targeting of proteins into peroxisomes.

Materials and methods

Reagents

The anti-CAT producing hybridoma line was a gift from C.Gorman (Genentech, San Francisco, CA). The anti-thiolase antibodies (Bodnar and Rachubinski, 1990) and anti-SKL antibodies (Gould *et al.*, 1990b) have been described previously. The CV-H Px110 cell line was obtained by hygromycin selection of CV-1 cells co-transfected with pSV2−CATC−PMP20 (Gould *et al.*, 1990a) and pTK−Hyg (Sugden *et al.*, 1985). The CV-H Px110 cell line expresses the CAT−PMP20 fusion protein in ~50% of the cells. All other reagents were obtained from standard sources.

Plasmids

All manipulations of DNA were performed essentially as described by Sambrook *et al.* (1987). We used pRSV−An for the expression of the thiolase gene constructs described below. In essence pRSV−An is pRSV−CAT (Gorman *et al.*, 1982a) in which the CAT containing *Hin*dIII−*Bam*HI fragment is replaced with sequences derived from the

*Bcl*I−*Bam*HI SV40 fragment (positions 2770−2533) containing the early region polyadenylation signal (An). Between the RSV promoter and the SV40 An signal pRSV−An contains a *Hin*dIII, an *Xba*I and a *Bgl*II site for cloning. The incomplete rat thiolase 2 (or A) cDNA clone pT4-4 (Bodnar and Rachubinski, 1990), lacking about 180 bp at its 5′ end, was used as template in a PCR with the primers SGThiolase 5′-GGA AGC TTA CCA TGG CTA CCT TCC CGC AGG C-3′ and Thiolase-2 5′-GAG ATC TAG TTC CCA GGG TAT TCA AAG ACA GC-3′. The fragment obtained by this PCR (~1100 bp) was digested with *Hin*dIII and *Bgl*II and cloned into pRSV−An digested with *Hin*dIII and *Bgl*II to obtain pRSV−thiol, encoding a protein corresponding to the mature thiolase. The internal *Sfi*I−*Stu*I fragment in pRSV−thiol was replaced with that isolated from pT4-4 to exclude possible mutations resulting from PCR from most of the thiolase gene. This clone, pRSV−thiol-40, was used for further experiments. The cDNA clone p5-6 (Bodnar and Rachubinski, 1990), containing the 5′ end of thiolase 2 (or A) cDNA, was used as template in a PCR with primers FLT-1 5′-GGG AAG CTT GCG GCC GCC ATT TTG GTT TGT TAA GC-3′ and INT-1 5′-CCC TCT AGA GGG CCC GCG CGG CCG ATG GGG GTG CG-3′. The fragment obtained by this PCR (~240 bp) was digested with *Hin*dIII and *Xba*I and cloned into pTZ-19U (Pharmacia-LKB) digested with *Hin*dIII and *Xba*I. A *Hin*dIII−*Sfi*I fragment containing the thiolase A 5′-end was isolated from this plasmid (pTZ−FLT1) and was cloned into pRSV−thiol-40 digested with *Hin*dIII and *Sfi*I to give pRSV−FLT1−thiol, encoding thiolase A. The thiolase A cDNA contains a *Hin*fI site downstream of its initiation codon but upstream of its second methionine codon, which corresponds to the initiation codon of the thiolase B gene. We used this *Hin*fI site to remove the thiolase A initiation codon from pTZ−FLT1 to obtain pTZ−FLT2 which contains a 5′ end corresponding to that of the thiolase B gene. A *Hin*dIII−*Sfi*I fragment was isolated from pTZ−FLT2 and cloned into pRSV−thiol-40 to obtain pRSV−FLT2−thiol, encoding thiolase B. pSV2−Not-leader−CAT consists of pSV2−CAT (Gorman *et al.*, 1982b) with a *Not*I site and several other sites from the pUC19 polylinker immediately upstream of the CAT translational initiation site. This allows the in-frame fusion of the amino-terminal parts of thiolases A and B to the CAT protein. pTZ−FLT1 and pTZ−FLT2 were both digested with *Not*I and *Xba*I to release fragments encoding the 5′-ends of the thiolases A (~230 bp) and B (~190 bp), respectively. These fragments were cloned into pSV2−Not-leader−CAT digested with *Not*I and *Xba*I to obtain pSV−FLT1−CAT and pSV−FLT2−CAT, respectively. We used PCR to fuse deletions in the thiolase B prepiece onto CAT. pSV2−CAT was used as template in a PCR with primers TCF-1 5′-CTG GCC GGC CGC TCG AGT GGT ACC ATG GAG AAA AAA ATC ACT GGA-3′ and CAT-3 5′-ATG GAT CCA GAT CTT ACG CCC CGC CCT GCC-3′. The fragment obtained by this PCR (~700 bp) was digested with *Eag*I and *Bgl*II and cloned into pRSV−FLT2−thiol digested with *Eag*I and *Bgl*II to obtain pRSV−FLT3−CAT (see Figure 3B). pRSV−thiol-40 was digested with *Bgl*II and partially with *Nco*I to release a fragment of ~1100 bp encoding the mature thiolase. This fragment was cloned into pRSV−FLT3−CAT digested with *Nco*I and *Bgl*II to obtain pRSV−FLT3−thiol. The 700 bp fragment obtained in the TCF-1/CAT-3 PCR was diluted 10 000× and used as template in a PCR primed with FLT-4 5′-GCC TGC AGG TAG TGC TGG GCC ACC TCT CGA GTG GTA CCA TGG-3′ and CAT-3. The fragment obtained by this PCR (~700 bp) was digested with *Pst*I and *Bam*HI and cloned into pTZ−FLT2 digested with *Pst*I and *Bam*HI to obtain pTZ−FLT4−CAT. This plasmid was digested with *Hin*dIII and *Bgl*II to release the FLT4−CAT fragment that was cloned into pRSV−An digested with *Hin*dIII and *Bgl*II to obtain pRSV−FLT4−CAT. For the construction of pRSV−FLT5−CAT we also used the diluted TCF-1/CAT-3 PCR fragment as template in a PCR, this time primed with FLT-5 5′-GGA AGC TTG CCA TGC ATC GCC TGC AGT CGA GTG GTA CCA TGG-3′ and CAT-3. The fragment obtained by this PCR (~700 bp) was digested with *Hin*dIII and *Bgl*II and cloned into pRSV−An digested with *Hin*dIII and *Bgl*II to obtain pRSV−FLT5−CAT.

Mammalian transfections

CV-1 and CVH Px110 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).

Immunofluorescence microscopy

Immunofluorescence was performed essentially as described earlier (Keller *et al.*, 1987). CV-1 or CVH Px110 cells were transfected with plasmids as indicated. 48 h after transfection cells were washed and fixed as described. In most experiments the cells were permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes, but for the digitonin-permeabilization experiments, a treatment of 25 μg/ml digitonin in PBS or 25 μg/ml digitonin plus 1% Triton X-100 was used. CV-H Px110 cells, transfected with one of the thiolase constructs were incubated with rabbit anti-thiolase antibodies and the CAT2 mouse monoclonal anti-CAT antibody, followed by a fluorescein conjugate of a goat anti-rabbit IgG antibody and a rhodamine conjugate of a goat anti-mouse IgG antibody. CV-1 cells transfected with pSV2CAT or one of the CAT-containing fusion proteins were incubated with the CAT2 mouse monoclonal anti-CAT antibody and with the rabbit anti-SKL antibodies, followed by a fluorescein conjugate of a goat anti-mouse IgG antibody and a rhodamine conjugate of a goat anti-rabbit IgG antibody.

Western blotting

Cells transfected with pRSV−FLT2−thiol, pRSV−FLT3−thiol, or pRSV−thiol-40 were harvested after 48 h incubation, lysed in SDS−PAGE sample buffer, separated by SDS−PAGE, and transferred to nitrocellulose essentially as described by Sambrook *et al.* (1987). The filter was treated with 0.05% Tween 20 in Tris-buffered saline (TBS) and then incubated with rabbit anti-thiolase antibodies for 4 h. The nitrocellulose filter was washed with TBS several times, incubated with [^{125}I]protein A and again washed with TBS. The filter was then exposed to Kodak XAR-5 X-ray film for autoradiography.

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