

Targeting of Human Catalase to Peroxisomes Is Dependent upon a Novel COOH-Terminal Peroxisomal Targeting Sequence

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Abstract. We have identified a novel peroxisomal targeting sequence (PTS) at the extreme COOH terminus of human catalase. The last four amino acids of this protein (-KANL) are necessary and sufficient to effect targeting to peroxisomes in both human fibroblasts and *Saccharomyces cerevisiae*, when appended to the COOH terminus of the reporter protein, chloramphenicol acetyl transferase. However, this PTS differs from the extensive family of COOH-terminal PTS tripeptides collectively termed PTS1 in two major aspects. First, the presence of the uncharged amino acid, asparagine, at the penultimate residue of the human catalase PTS is highly unusual, in that a basic residue at this position has been previously found to be a common and critical feature of PTS1 signals. Nonetheless, this asparagine residue appears to constitute an important component of the catalase PTS, in that replacement with aspartate abolished peroxisomal targeting (as did deletion of the COOH-terminal four residues). Second, the human catalase PTS comprises more than the COOH-terminal three amino acids, in that COOH-terminal -ANL cannot functionally replace the PTS1 signal -SKL in targeting a chloramphenicol acetyl transferase fusion protein to peroxisomes. The critical nature of the fourth residue from the COOH terminus of the catalase PTS (lysine) is emphasized by the fact that substitution of this residue with a variety of other amino acids abolished or reduced peroxisomal targeting. Targeting was not reduced when this lysine was replaced with arginine, suggesting that a basic amino acid at this position is required for maximal functional activity of this PTS. In spite of these unusual features, hu-

man catalase is sorted by the PTS1 pathway, both in yeast and human cells. Disruption of the *PAS10* gene encoding the *S. cerevisiae* PTS1 receptor resulted in a cytosolic location of chloramphenicol acetyl transferase appended with the human catalase PTS, as did expression of this protein in cells from a neonatal adrenoleukodystrophy patient specifically defective in PTS1 import. Furthermore, through the use of the two-hybrid system, it was demonstrated that both the *PAS10* gene product (Pas10p) and the human PTS1 receptor can interact with the COOH-terminal region of human catalase, but that this interaction is abolished by substitutions at the penultimate residue (asparagine-to-aspartate) and at the fourth residue from the COOH terminus (lysine-to-glycine) which abolish PTS functionality. We have found no evidence of additional targeting information elsewhere in the human catalase protein. An internal tripeptide (-SHL-, which conforms to the mammalian PTS1 consensus) located nine to eleven residues from the COOH terminus has been excluded as a functional PTS. Additionally, in contrast to the situation for *S. cerevisiae* catalase A, which contains an internal PTS in addition to a COOH-terminal PTS1, human catalase lacks such a redundant PTS, as evidenced by the exclusive cytosolic location of human catalase mutated in the COOH-terminal PTS. Consistent with this species difference, fusions between catalase A and human catalase which include the catalase A internal PTS are targeted, at least in part, to peroxisomes regardless of whether the COOH-terminal human catalase PTS is intact.

CATALASE (EC 1.11.1.6) is a homotetrameric heme-containing enzyme present within the matrix of all peroxisomes, where it is responsible for degradation to water and oxygen of the hydrogen peroxide gener-

ated through the action of peroxisomal oxidases (13, 33, 62). The fundamental biochemical importance of this activity is exemplified by the observation that catalase-deficient yeast cells fail to grow on fatty acid substrates of peroxisomal β -oxidation, presumably due to accumulation of toxic hydrogen peroxide (65, 72). Moreover, the pivotal role of catalase in peroxisomal biology is underscored by its assignment as a defining characteristic of peroxisomes, which otherwise demonstrate considerable variation in

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biochemical pathways between different tissues and species (13, 33, 62).

The biogenesis of catalase exhibits a number of interesting features. As for all peroxisomal proteins (33), catalase is synthesized on cytosolic free polyribosomes (22, 49). In rat liver, import of catalase monomers has been shown to occur posttranslationally with a half-life of 14 min. After import, addition of heme and tetramerization proceed within the organelle to produce mature active catalase (32). However, there is considerable evidence that catalase assembly can also occur outside of the peroxisome. For instance, in both guinea pig liver (71) and yeast cells (59, 61), catalase activity is present both in peroxisomes and in the cytosol. Furthermore, in cells of patients with Zellweger Syndrome, in which catalase (and other peroxisomal matrix proteins) are synthesized normally, but fail to be imported across the peroxisomal membrane (34, 62), catalase assembles into catalytically active tetramers in the cytosol (31, 52, 66, 67). Similarly, in yeast peroxisome biogenesis mutants, catalase A (normally peroxisomal) assembles to its active form in the cytosol (18, 19, 65, 72). Recent observations raise the question as to whether peroxisomal catalase is targeted to the organelle exclusively as monomers (41). An investigation which used monoclonal antibodies specific for tetrameric and dimeric/monomeric catalase subunits concluded that in contrast to the situation in rodent liver, human skin fibroblasts assemble cytosolic tetrameric catalase within the cytosol (within an hour after synthesis), which is then targeted to peroxisomes for disassembly and import (42). Corroborating evidence that tetrameric catalase retains import competence comes from complementation analysis of Zellweger Syndrome cell lines. These cells can be divided into distinct complementation groups such that fusion of cells from different groups results in appearance within the hybrids of catalase-containing peroxisomes (8, 54, 70). In some cases, this process is cycloheximide-insensitive, implying that the packaged catalase is derived from the assembled cytosolic catalase present in the mutants before fusion (9). The extensive analysis of catalase targeting in the characterization of cell lines from patients with Zellweger syndrome and related disorders provides a clear rationale for determination of the catalase PTS, without which the interpretation of such targeting data is difficult.

To better understand the biogenesis pathway of peroxisomal catalase, recent effort has been devoted to the identification of catalase peroxisomal targeting sequences (PTSs),¹ through the expression and subcellular localization of various catalase mutants and fusion proteins generated by in vitro mutagenesis. Of particular interest is the identification of two independent PTSs in catalase A of *Saccharomyces cerevisiae*, one located internally and the other at the COOH terminus (30), and the identification of a PTS within the COOH-terminal 27 residues of human catalase (25). These 27 amino acids are of special interest in that they terminate with a sequence (-ANL) that falls outside the defined consensus for mammalian COOH-terminal tripeptide PTSs, but do contain an internal tripep-

ptide (-SHL-), nine to eleven residues from the COOH terminus, which does conform to this consensus (25). This prompted speculation that tripeptide PTSs might be able to function at internal locations in some instances (25). Also of interest is the observation that even though the COOH-terminal end of human catalase shows no significant sequence homology to that of yeast catalase A, the transgenically expressed human protein is targeted to yeast peroxisomes in vivo (14), implying evolutionary conservation of the peroxisomal sorting of this protein. In this paper, we address the molecular basis of this conservation of targeting by analyzing the sequences within human catalase responsible for targeting of this protein to yeast and human peroxisomes.

Materials and Methods

Oligonucleotides

The sequences of the oligonucleotides used in this study as polymerase chain reaction (PCR) primers and for in vitro mutagenesis were as follows: C2, 5'-GGCAAGGGAGTAGGCAAATC-3'; C3, 5'-TCCGGAACTCAGGTGGCGGCA-3'; C4, 5'-GAGAAGGCAGATCTGTGAGG-3'; C5, 5'-CGGCAAGGGAGGGGGCAAATCTGTGA-3'; A1, 5'-AAG-ATGTCGAAATTGGGACAAGAA-3'; A2, 5'-GAAAACTCGAGATGCATATGGACA-3'; CH1, 5'-CTTGGCGAGATTTTCAGGAGC-3'; CH2, 5'-CTAGAGATTCGCCTTCTCCCTTGCCGCAACGCCCGCCCTG-3'; CH3, 5'-CTAGAGATTCGCCTTCTCGGCGCCGCCCTG-3'; CH4, 5'-CTAGAGATTCGCCTTGGCGCCGCCCTG-3'; CH5, 5'-CTAGAGATTCGCGCGCCGCCCTG-3'; CH6, 5'-CTAGAGATTCGCTCCCGCCCGCCCTG-3'; CH7, 5'-CTAGAGCTTCGATCCCGCCCGCCCTG-3'; CH8, 5'-CTAGAGATTCGCNNTCCGGCGCCGCCCTG-3'. C2-C5 were used for site-directed mutagenesis of the 3' end of the cDNA encoding human catalase. C2 corresponds to nucleotides 1560-1579 of the human catalase cDNA coding sequence (5), and contains a single mismatch (underlined) to convert a lysine codon (AAG) to a termination codon (TAG). C3 (nucleotides 1543-1563) contains three mismatches (underlined) to convert the nonanucleotide encoding SHL (TCT CAC TTG) to one encoding TQV (ACT CAG GTG). C4 (nucleotides 1567-1586) contains a single mismatch (underlined) to convert an asparagine codon (AAT) to an aspartate codon (GAT). C5 (nucleotides 1559-1584) contains two mismatches (underlined) to convert a lysine codon (AAG) to a glycine codon (GGG).

A1 and A2 were used as PCR primers to amplify the 5' region of the yeast catalase A gene. A1 corresponds to the start of the coding region (ATG initiation codon underlined), and A2 is complementary to nucleotides 1124-1143 of the coding sequence (12), and includes three mismatches (CTAAAT → CTCGAG) to introduce an XhoI site (underlined) equivalent to that present in the human catalase coding sequence (5).

CH1 and each of CH2-CH8 were used to amplify the complete coding region of the chloramphenicol acetyl transferase gene from the plasmid pCAT-Basic (Promega, Madison, WI). CH1 maps from nucleotides -34 to -14 relative to the start of the open reading frame. CH2-CH8 are complementary to the 3' end of the gene, modified to extend the sequence by -LAAREKANL (CH2), -EKANL (CH3), -KANL (CH4), -ANL (CH5), -GANL (CH6), -GSKL (CH7), and -EXANL, where X is any amino acid, or a termination codon (CH8).

PCR

One microgram of yeast genomic DNA was subjected to PCR amplification using primers A1 and A2. Amplification buffer consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs, and 2 μM of each primer. Samples were heated to 95°C for 5 min before addition of 2.5 U of Taq Polymerase (Boehringer Mannheim Corp., Mannheim, FRG). Thirty five cycles of 95°C (90 s), 55°C (60 s), 72°C (120 s), were followed by a final incubation at 72°C for 10 min. For amplification of plasmid pCAT-Basic with primers CH1-CH8, conditions were the same, except that the template was 10 ng plasmid DNA, and the 72°C extension time was reduced to 60 s.

1. Abbreviations used in this paper: AGT, alanine:glyoxylate aminotransferase; ChAT, chloramphenicol acetyl transferase; PTS, peroxisomal targeting sequence.

Construction of Plasmids

The plasmids used in this study are shown in Table I. Construction was as follows:

phCAT_{mut} (for in vitro mutagenesis of human catalase cDNA) was generated by subcloning a PstI–PstI fragment containing the complete catalase cDNA coding region from pCAT10 (contains full-length catalase cDNA [29] in pSP65 vector [Promega]) into the PstI site of pSELECT (Promega).

phCAT1 was constructed by subcloning catalase cDNA from pCAT10 as a PstI–PstI fragment (see above) into the PstI site of pBluescriptKS(+) (Stratagene, La Jolla, CA) to produce a clone with the catalase cDNA on a Sall–Sall fragment, which was then excised and cloned into XhoI digested pCUP1, such that expression of the human catalase is under the control of the copper-sensitive CUP1 promoter (10, 20).

phCAT2, phCAT3, and phCAT4 were constructed by site-directed mutagenesis of phCAT_{mut} with oligonucleotides C2, C3, and C4, respectively, followed by replacement of the catalase cDNA 3' coding region of phCAT1 with the appropriate mutated sequences using XhoI (cuts uniquely within the cDNA coding region of phCAT1) and SacI (cuts 3' to the cDNA coding region of phCAT1).

pCATA-hCAT1 and pCATA-hCAT4 were constructed by replacing the 5' regions of phCAT1 and phCAT4, respectively, with the equivalent region of the *S. cerevisiae* catalase A gene. This was achieved by PCR amplifying yeast genomic DNA with primers A1 and A2 (see above) in order to introduce an XhoI restriction site at the equivalent position to the unique XhoI site of human catalase cDNA. The PCR product was then cloned into pT7Blue (Novagen, Madison, WI), and re-excised as an AccI–XhoI fragment, which was then used to replace the ClaI–XhoI fragment (which specifies the 5' region of human catalase cDNA) of phCAT1 and phCAT4.

pChAT-hCAT9 was constructed by cloning the CH1/CH2 PCR product from pCAT-Basic into the SmaI site of pT7Blue, and then subcloning (using Sau3A) into pPOX1 (which contains the promoter of yeast acyl Co-A oxidase cloned into pRS316 [55]). pChAT-EKANL, pChAT-KANL, and pChAT-ANL were constructed in exactly the same manner, except using CH1/CH3, CH1/CH4, and CH1/CH5 PCR product, respectively. For the construction of a series of plasmids identical to pChAT-EKANL, but with the lysine residue near the COOH terminus replaced with various other residues, the procedure was the same, but using PCR product generated with CH1 and the degenerate oligonucleotide CH8. Clones of interest were selected by sequencing the pT7Blue recombinants before subcloning into pPOX1. pChAT-GANL and pChAT-GSKL were constructed in a similar way, except that the CH1/CH6 and CH1/CH7 PCR products from pCAT-Basic were cloned into the SmaI site of pRS314 (55), before subcloning into pPOX1, using EcoRV and BamHI.

pCMVChAT-GSKL, pCMVChAT-GANL, pCMVChAT-KANL, and pCMVChAT-hCAT9 were constructed by subcloning the respective cloned PCR products into pCMV-link, a vector constructed by the insertion of the pBluescriptKS(+) polylinker into pCMV β (35), as replacement for the β -galactosidase gene. The cloning enzymes were NotI and KpnI (pCMVChAT-GSKL and pCMVChAT-GANL), and NotI and SpeI (pCMVChAT-KANL and pCMVChAT-hCAT9).

pGBT9-hCAT1 and pGBT9-hCAT4 were constructed by cloning the XhoI–PstI fragments (which encode the COOH-terminal portion of human catalase) of phCAT1 and phCAT4, respectively, into pGBT9 (Clontech Laboratories, Palo Alto, CA) to create in-frame fusions between the GAL4 DNA-binding domain and catalase. pGBT9-hCAT1KG was generated by site-directed mutagenesis of pGBT9-hCAT1 with primer C5. These plasmids were used as partners in the two-hybrid system with pGAD424-pas10 and pPTS1-BP (21). These latter clones encode the yeast *PAS10* and human *PTS1* receptor genes cloned in pGAD10 and pGAD424 (Clontech Laboratories), respectively, to create in-frame fusions with the GAL4 transactivation domain.

pCATA-URA3, used for disruption of the catalase A gene from strain 3A, was constructed by excising the URA3 gene from plasmid pAC100.2 as an XbaI–XbaI fragment, blunt-ending with Klenow, and inserting into the EcoRV site within the catalase A coding region of cloned A1/A2 PCR product (described above in the construction of pCATA-hCAT1 and pCATA-hCAT4).

Yeast Strains

Our laboratory "wild-type" strain 3A (*MAT α* , *ura3-1*, *trp1-1*, *arg4*, *ctl1-1*) (JW68-3A in [72]) is deficient in cytosolic catalase T, but does contain peroxisomal catalase A. Strain 3A- Δ CatA (*MAT α* , *ura3-1*, *trp1-1*, *arg4*, *ctl1-1*,

Table I. Plasmids Used in This Study

Name	Description
pCAT10	Full-length human catalase cDNA cloned into EcoRI site of pSP64.
phCAT _{mut}	Complete human catalase cDNA coding sequence cloned into pSELECT
phCAT1	Complete human catalase cDNA coding sequence cloned into pCUP1
phCAT2	phCAT1, mutated to encode catalase lacking the COOH-terminal 4 residues
phCAT3	phCAT1, mutated to encode catalase with internal -SHL- changed to -TQV-
phCAT4	phCAT1, mutated to encode catalase with COOH-terminal -ANL changed to -ADL
pCATA-hCAT1	phCAT1, with region encoding human catalase residues 1-383 replaced with the portion of <i>S. cerevisiae</i> catalase A gene encoding residues 1-378
pCATA-hCAT4	phCAT4, with region encoding human catalase residues 1-383 replaced with the portion of <i>S. cerevisiae</i> catalase A gene encoding residues 1-378
pChAT-hCAT9	Chloramphenicol acetyl transferase gene, modified to encode an additional nonapeptide corresponding to the human catalase COOH terminus, under control of the promoter of the <i>S. cerevisiae</i> POX1 gene, cloned into pRS316.
pChAT-EKANL	pChAT-hCAT9, but with the COOH-terminal nonapeptide replaced with -EKANL
pChAT-KANL	pChAT-hCAT9, but with the COOH-terminal nonapeptide replaced with -KANL
pChAT-ANL	pChAT-hCAT9, but with the COOH-terminal nonapeptide replaced with -ANL
pChAT-GANL	pChAT-hCAT9, but with the COOH-terminal nonapeptide replaced with -GANL
pChAT-GSKL	pChAT-hCAT9, but with the COOH-terminal nonapeptide replaced with -GSKL
pChAT-EXANL	pChAT-hCAT9, but with the COOH-terminal nonapeptide replaced with -EXANL, where X is one of several different amino acids
pCMVChAT-hCAT9	Chloramphenicol acetyl transferase gene, modified to encode an additional nonapeptide corresponding to the human catalase COOH-terminus, under control of the CMV promoter in a human expression vector based on pCMV β .
pCMVChAT-GANL	pCMVChAT-hCAT9, but with the COOH-terminal nonapeptide replaced with -GANL
pCMVChAT-GSKL	pCMVChAT-hCAT9, but with the COOH-terminal nonapeptide replaced with -GSKL
pCMVChAT-KANL	pCMVChAT-hCAT9, but with the COOH-terminal nonapeptide replaced with -KANL
pGAD424-pas10	GAL4 activation domain fused to Pas10p, under control of ADH promoter.
pPTS1-BP	GAL4 activation domain fused to amino acids 258-639 of human PTS1 receptor, under control of ADH promoter (from Dr. M. Fransen).
pGBT9-hCAT1	GAL4 DNA-binding domain fused to residues 382-527 of normal human catalase, under control of ADH promoter.
pGBT9-hCAT4	pGBT9-hCAT1, but with COOH-terminus of fusion protein mutated to -KADL.
pGBT9-hCAT1KG	pGBT9-hCAT1, but with COOH-terminus of fusion protein mutated to -GANL.
pCATA-URA3	URA3 gene cloned within catalase A coding region. For catalase A disruption.

cat1::URA3), which is deficient in both catalase A and catalase T, was constructed by disruption of the catalase A gene from 3A. This was effected by transformation of 3A with KpnI digested pCATA-URA3 followed by selection of Ura⁺ clones, which were screened by immunofluorescence and immunoblotting with anti-catalase A. Strain SFY526 (*MAT α* , *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *112*, *can1*, *gal4-542*, *gal80-538*) (4) contains a *GAL1-lacZ* gene fusion suitable for use as a reporter for the two-hybrid system (11). Strain 3A- Δ *pas10* (*MAT α* , *ura3-1*, *trp1-1*, *arg4*, *ctt1-1*, *pas10::TRP1*), kindly supplied by Dr. J.W. Zhang (Mount Sinai School of Medicine, New York), will be described in detail elsewhere. This strain is disrupted at the *PAS10* locus.

Yeast Growth Conditions

For induction of peroxisomes, cells were grown as previously described (72), except that preculturing was routinely performed in SD medium (0.67% yeast nitrogen base, 2% dextrose) supplemented with uracil and appropriate amino acids, to maintain selective pressure on plasmids introduced into the yeast cells, before growth in YPGO medium (1% yeast extract, 2% peptone, 3% glycerol, 0.1% oleic acid, 0.25% Tween 40) for 18 h. Expression from the copper-sensitive CUP1 promoter (10, 20) was found to be significant in the absence of added copper, and only moderately enhanced by copper addition (48). Therefore, copper was not added to the YPGO for the culturing of cells containing plasmids with the CUP1 promoter.

For the two-hybrid system, cells were grown to mid-log phase in SD medium supplemented with adenine and appropriate amino acids before glass-bead homogenization.

Transformation and Transfection of Human Fibroblasts

Normal human axillary skin fibroblasts, and fibroblasts from a neonatal adrenoleukodystrophy patient (patient A from complementation group 2 in [44], also referred to as patient PBD018 in [16]) were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% FCS. For transformation, 10⁴ cells were trypsinized with 0.025% trypsin in phosphate-buffered saline (PBS), and seeded at a 1:5 dilution into 60-mm petri dishes. When subconfluency was reached, cells were washed twice with PBS, and incubated with recombinant SV40-Adenovirus (17) for 3 h in serum-free medium with occasional shaking. Cells were then washed twice with PBS and cultured in MEM supplemented with 4% FCS, with medium being changed every 3 d. After three weeks, colonies were picked and dispersed into MEM with 4% FCS in small petri dishes. Transformation was confirmed by morphological changes, proliferative capacity, and detection of SV40 large T antigen by immunofluorescence. Calcium phosphate transfection, without DMSO or glycerol shock, was as described (3).

Immunofluorescence

Immunofluorescence analysis of YPGO-induced yeast cells was performed essentially as described previously (72), with the exception that spheroplast formation was performed at 37°C for 15 min in SP buffer (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4) supplemented with 30 μ g/ μ l Zymolyase and 3 μ l/ml β -mercaptoethanol. Affinity-purified 10-330, a polyclonal rabbit antibody against bovine liver catalase which cross-reacts with human catalase (51), was used at a dilution of 1:5. Polyclonal rabbit anti-chloramphenicol acetyl transferase IgG (5'-3' Inc., Denver, CO) and goat antiserum against catalase A (a kind gift of Dr. Andreas Hartig, University of Wien, Wien, Austria) were used at 1:100-1:200. Rabbit antiserum against thiolase (a kind gift of Dr. Wolf Kunau, University of Bochum, Bochum, Germany) was used at 1:400. The secondary antibodies (Amersham International, UK) were goat anti-rabbit IgG conjugated to FITC, used at a dilution of 1:500 (for 10-330, anti-chloramphenicol acetyl transferase, and anti-thiolase), and mouse anti-goat IgG conjugated to FITC, used at a dilution of 1:64 (for anti-catalase A). For double immunofluorescence with anti-chloramphenicol acetyl transferase and anti-catalase A, a mixture of the primary antibodies at dilutions of 1:100 each was applied first, followed by donkey anti-rabbit IgG coupled to FITC (1:50 dilution) and then, after removal of the secondary antibody and thorough washing, rabbit anti-goat IgG, coupled to TRITC (1:160 dilution). The specificity under these conditions of each of the secondary antibodies was confirmed through control immunofluorescence reactions in which the primary antibodies were used separately.

Immunofluorescence of transfected fibroblasts was essentially as described previously (51), except that permeabilization was with 1% NP-40

instead of methanol, and postfixation washes were once in 0.1 M NH₄Cl in PBS, followed by once in PBS. Polyclonal anti-chloramphenicol acetyl transferase was used at a dilution of 1:400.

Immunoelectron Microscopy

Electron microscopic immunolocalization of catalase with gold particles was as described by Wright and Rine (69), using polyclonal 10-330 antiserum at a dilution of 1:100, and protein A-gold at 1:50. The size of the gold was 20 nm.

Other Methods

Standard techniques of molecular biology (3, 50) and yeast genetics (53) were used throughout. DNA sequencing, used to confirm the identity of various DNA constructs, was by the dideoxy method, with modified T7 DNA polymerase (Sequenase II, United States Biochem. Corp., Cleveland, OH). In vitro site-directed mutagenesis was performed using the Altered Sites system (Promega), or the Transformer system (Clontech Laboratories) as recommended by the manufacturers. Subcellular fractionation and glass bead homogenization of yeast cells were as described previously (61), and immunoblotting was performed with the ECL chemiluminescence system (Amersham International), using 1:4,000 dilutions of polyclonal 10-330 serum, anti-catalase A and anti-chloramphenicol acetyl transferase, and 1:10,000 dilutions of anti-thiolase and anti- β -galactosidase.

Materials

Anti- β -galactosidase was from Promega. Plasmids pCAT10, pAC100.2, pPTS1-BP, and pGAD424-pas10 were generously supplied by Dr. Roy Gravel (University of Amsterdam), Dr. Jeanne Hirsch (Mount Sinai School of Medicine, New York), Dr. Marc Franssen (University of Leuven), and Dr. Jing-Wei Zhang (Mount Sinai School of Medicine, New York), respectively. Restriction nucleases and modifying enzymes were from Promega or Boehringer Mannheim.

Results

Human Catalase Is Targeted to Yeast Peroxisomes

The ability of yeast peroxisomes to import human catalase was initially assessed by analysis of wild-type strain 3A (see Materials and Methods) transformed with plasmid phCAT1, which contains normal human catalase cDNA under the regulation of the copper-sensitive CUP1 promoter. Immunofluorescence analysis of these cells with affinity-purified polyclonal 10-330 (which was previously raised against bovine liver catalase, and recognizes human catalase [51]) revealed a punctate pattern of human catalase distribution that was not seen with untransformed cells (Fig. 1). That this reflected peroxisomal targeting of the human catalase was verified by immunoelectron microscopic analysis of 3A-phCAT1 with 10-330 antibody. Labeling with gold particles was effectively confined to the peroxisomal matrix (Fig. 2 A). No other portion of the cells was labeled to any significant extent. Differential centrifugation analysis of this strain demonstrated that most of the transgenic human catalase was in the organellar pellet (Fig. 3). These results clearly show that human catalase is imported into yeast peroxisomes efficiently. This import is independent of the biogenesis of the endogenous yeast catalase A, since it was unaffected by disruption of the host 3A catalase A gene (Fig. 3).

Mutational Strategy

To determine the sequence(s) of human catalase functioning as a peroxisomal targeting sequence (PTS) in yeast,

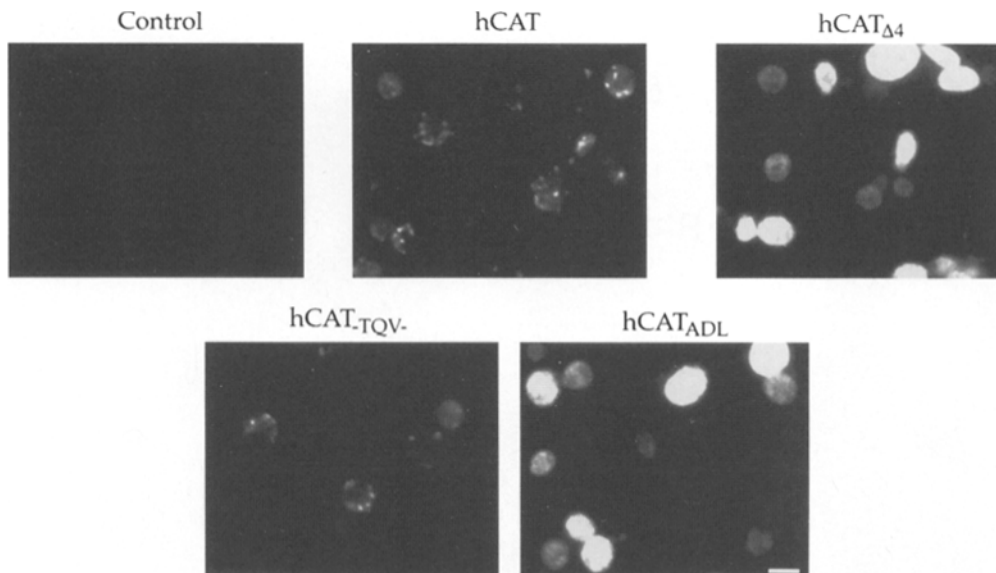


Figure 1. Packaging of human catalase (and variants) into yeast peroxisomes, as assessed by immunofluorescence. 3A cells were transformed with plasmids encoding normal human catalase (hCAT; plasmid pHCAT1), human catalase with the COOH-terminal four amino acids deleted (hCAT Δ 4; plasmid pHCAT2), human catalase with the internal -SHL-tripeptide changed to -TQV- (hCAT $_{TQV}$; plasmid pHCAT3), and human catalase with the penultimate residue changed from asparagine to aspartate (hCAT $_{ADL}$; plasmid pHCAT4). Control, untransformed 3A. The primary antibody was polyclonal 10-330, which identifies human catalase, and the secondary antibody was goat anti-

rabbit IgG, coupled to FITC. The variation in intensity of fluorescence between cells in this figure, and in Figs. 4, 5, and 7, reflects the fact that certain cells lie outside the focal plane, as well as the occasional loss of plasmid from some cells during peroxisome induction on YPGO medium. The consistent observation that cells with cytosolic fluorescence appear to give a stronger signal than those with punctate fluorescence (see also Figs. 4 and 5) represents an unexplained feature of our immunofluorescence experiments. It is not due to variation in the level of expression of the introduced plasmid (see immunoblots in Figs. 3 and 4). Bar, 5 μ m.

various mutations were introduced into the catalase cDNA sequence before expression in 3A cells. Since the region of human catalase responsible for peroxisomal targeting in mammalian cells had previously been suggested to be limited to the COOH-terminal 27 residues (25), it was this region of the protein that was selected for modification. Two tripeptides within this region were of immediate interest. One of these was the COOH-terminal tripeptide, which, by analogy with numerous other peroxisomal proteins, is a likely location for a PTS. However, the uncharged asparagine in the middle of this tripeptide (-ANL) excludes it from the defined consensus for PTS activity in mammalian cells (S/A/C - K/R/H - L), which always has a positively charged amino acid at the penultimate position. This heightened interest in the second significant region, an internal tripeptide (-SHL-) located nine-to-eleven residues from the COOH terminus, which does conform to the aforementioned consensus (23).

Targeting Requires the Extreme COOH-Terminal Sequence

A clone encoding catalase with a deletion of the COOH-terminal four amino acids under the regulation of the CUP1 promoter (pHCAT2; see Materials and Methods and Table I) was constructed and transformed into strain 3A. Immunofluorescence analysis revealed that the truncated catalase produced from pHCAT2 (hCAT Δ 4) was distributed throughout the cytosol, in contrast to the picture seen for normal catalase (Fig. 1). This was confirmed by immunoelectron microscopy, which showed an absence of peroxisomal decoration with gold, but an apparent increase in labeling over the cytosolic compartment (data similar to Fig. 2 B, not shown). A virtually identical set of results was found for catalase that contained a single

amino acid substitution (asparagine to aspartate) at the penultimate COOH-terminal residue (hCAT $_{ADL}$, encoded by plasmid pHCAT4). This mutated catalase was found in the cytosol by immunofluorescence (Fig. 1). It was missing from peroxisomes by immunogold labeling (Fig. 2 B). Furthermore, cell fractionation analysis confirmed that this single amino acid substitution effectively abolished targeting of the human catalase to peroxisomes, with only trace amounts of hCAT $_{ADL}$ being found in the organellar pellet, in contrast to the efficient import seen in the case of normal human catalase (Fig. 3). These results indicate that the targeting of human catalase to yeast peroxisomes requires the COOH-terminal four amino acids, and in particular depends upon the penultimate asparagine residue.

An Internal SHL Tripeptide Is Not Required for Targeting

To test whether the internal SHL tripeptide plays a role in the peroxisomal targeting of human catalase in yeast, a construct encoding catalase with this tripeptide changed to TQV (hCAT $_{TQV}$, encoded by pHCAT3) was generated, and then expressed in 3A cells. Immunofluorescence analysis of the transformed cells showed that, as for 3A-pHCAT1, catalase was localized in particles (Fig. 1). Furthermore, immunoelectron microscopy verified that the catalase-positive particles were peroxisomes (data similar to Fig. 2 A, not shown). These results indicate that the internal SHL tripeptide is not directly involved in peroxisomal targeting.

The COOH-Terminal Tetrapeptide of Human Catalase Is Sufficient for Targeting a Reporter Protein to Peroxisomes, but the COOH-Terminal Tripeptide Is Not

A clone (pChAT-hCAT9) encoding chloramphenicol acetyl

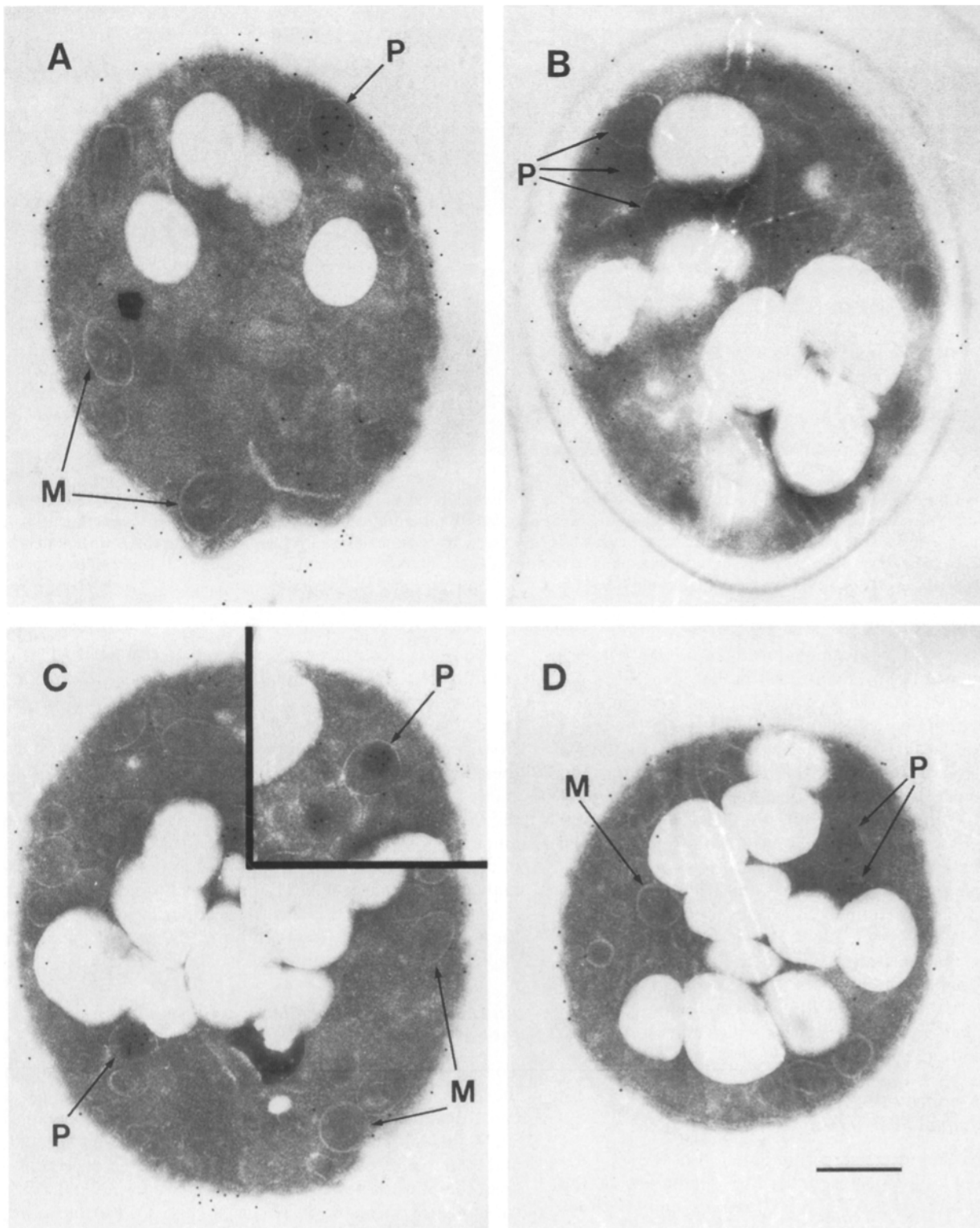


Figure 2. Subcellular localization of various forms of catalase expressed in yeast cells, as assessed by immunoelectron microscopy. 3A cells were transformed with (A) plasmid pHCAT1, encoding normal human catalase (hCAT); (B) plasmid pHCAT4 encoding human catalase with the penultimate residue changed from asparagine to aspartate (hCAT_{ADL}); (C) plasmid pCATA-hCAT1, encoding a fusion protein consisting of residues 1-378 of yeast catalase A fused to residues 384-527 of normal human catalase (CATA-hCAT); (D) plasmid pCATA-hCAT4, encoding an identical fusion protein to CATA-hCAT, but with the penultimate residue changed from asparagine to aspartate (CATA-hCAT_{ADL}). The inset in C, from a different cell at the same magnification, is shown to highlight the apparent intraperoxisomal aggregation of CATA-hCAT discussed in the text. The antibody was polyclonal 10-330, which identifies human catalase and the catalase A-human catalase fusion proteins (but not catalase A), followed by protein A-gold. This antibody, which had not been affinity-purified, also gives nonspecific labeling of the cell wall. Peroxisomes (P) can be clearly distinguished from mitochondria (M) by their single membrane and absence of cristae. Bar, 0.5 μ m.

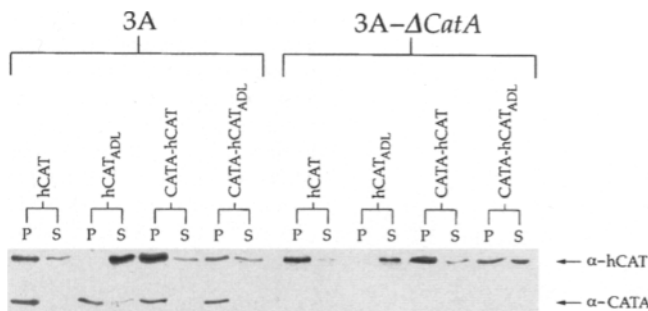


Figure 3. Immunoblot analysis of subcellular localization of various forms of catalase. 3A and 3A- Δ CatA were transformed with plasmids encoding normal human catalase (hCAT; plasmid pHCAT1), human catalase with the penultimate residue changed from asparagine to aspartate (hCAT_{ADL}; plasmid pHCAT4), a fusion protein consisting of residues 1-378 of yeast catalase A fused to residues 384-527 of normal human catalase (CATA-hCAT; plasmid pCATA-hCAT1), and an identical fusion protein to CATA-hCAT, but with the penultimate residue changed from asparagine to aspartate (CATA-hCAT_{ADL}; plasmid pCATA-hCAT4). Postnuclear supernatant fractions were separated by differential centrifugation (61) into organellar pellets (P), and high speed supernatants (S), which were then subjected to SDS-PAGE and immunoblotting. Antibody 10-330 (α -hCAT), which recognizes human catalase and the catalase A-human catalase fusion proteins (but not catalase A), was first used to detect the various transgenically expressed forms of catalase (upper portion of figure). After this, the blots were incubated with anti-catalase A (α -CATA), to detect the endogenous catalase A (lower portion of figure).

transferase (ChAT) appended with the last nine amino acids of human catalase (-LAAREKANL), under the regulation of the promoter of POX1 (which encodes peroxisomal acyl-CoA oxidase), was constructed and expressed in 3A yeast cells. Immunofluorescence analysis of this strain with antibody against ChAT revealed a punctate distribution pattern absent from untransformed 3A yeast (Fig. 4 A). Cell fractionation showed that the majority of this protein was present in the organellar pellet (Fig. 4 B). Double-immunofluorescence with anti-ChAT and goat anti-catalase A confirmed that the ChAT was targeted to peroxisomes (Fig. 4 C). To identify the minimal region within the human catalase COOH-terminal nonapeptide sufficient for peroxisomal targeting, a series of related plasmids with decreasing amounts of the human catalase COOH terminus appended to ChAT were constructed and expressed in 3A cells. This revealed that as few as the COOH-terminal four residues (-KANL) were sufficient for targeting to peroxisomes (Fig. 4 A, construct pChAT-KANL). However, further deletion of the lysine residue four amino acids from the COOH terminus (construct pChAT-ANL), or substitution of this lysine with glycine (pChAT-GANL), resulted in a totally different distribution pattern of ChAT, with the vast majority remaining in the cytosol, as assessed by both immunofluorescence and cell fractionation (Fig. 4, A and B). The unexpected implication of these results is that the human catalase PTS differs from other COOH-terminal PTSs in that it is defined not by three, but by four amino acids. This is emphasized by the observation that replacement of the human catalase COOH-terminal tripeptide of ChAT-GANL with the archetypal PTS1 signal,

-SKL (to create ChAT-GSKL), restored peroxisomal targeting (Fig. 4, A and B), a result which is in agreement with a previous report from McNew and Goodman (40), which showed that -SKL and -AKL were functional as PTSs in yeast when appended to the COOH terminus of ChAT via a single glycine residue. The ability of ChAT-GSKL (and ChAT-GAKL) to localize to peroxisomes effectively rules out the possibility that failure of ChAT-GANL to be targeted is due to the three-dimensional structure of this fusion protein rendering the COOH terminus inaccessible to the cellular machinery responsible for recognition and import of COOH-terminal PTSs.

A Positively Charged Amino Acid Four Residues from the COOH Terminus Is an Important Component of the Human Catalase PTS

Having established the importance of the residue fourth from the COOH terminus as part of the human catalase PTS, a more detailed analysis of the permissible amino acids at this position was conducted. For this purpose, in vitro mutagenesis was used to generate a series of clones identical to pChAT-EKANL (which encodes ChAT appended with the last five amino acids of human catalase), except with the lysine (underlined) replaced with one of a range of other amino acids. Expression of these plasmids in 3A cells followed by subcellular fractionation revealed that only arginine could replace this lysine without causing a major reduction in peroxisomal targeting (Fig. 5 A). Of the other amino acids tested in this position, most resulted in a dramatic reduction of peroxisomal targeting to levels ranging from undetectable to <10% peroxisomal (alanine, aspartate, asparagine, glycine and proline, methionine, and serine). However, other amino acids, such as leucine, glutamine, and valine resulted in only a moderate reduction in peroxisomal targeting (Fig. 5 A). Double immunofluorescence with anti-ChAT and goat anti-catalase A corroborated the cellular fractionation data. ChAT-ERANL showed strong colocalization with catalase A, consistent with efficient targeting of this construct to peroxisomes, whereas constructs which showed reduced targeting to the organellar pellet (such as ChAT-ELANL) also colocalized with catalase A to particles, but with a higher level of anti-ChAT cytosolic fluorescence than that seen with ChAT-ERANL, and constructs which were absent from the organellar pellet (such as ChAT-EGANL) gave only cytosolic fluorescence (Fig. 5 B). These results suggest that a basic amino acid, four residues from the COOH terminus of the human catalase COOH terminus, is required to maintain full PTS activity, but that a limited number of nonbasic residues at this position may also support sub-optimal PTS activity.

The *S. cerevisiae* PTS1 Receptor, Pas10p, Interacts with the COOH Terminus of Human Catalase, and Is Required for Human Catalase PTS Function

Pas10p, encoded by the *PAS10* gene, has been identified as the *S. cerevisiae* PTS1 receptor, partly by virtue of its homology to the well-characterized PTS1 receptor of *Pichia pastoris*, Pas8p (64), and partly by demonstration of its interaction with -SKL in the yeast two-hybrid system (7). We tested the interaction of Pas10p with the human catalase PTS. Cotransformation of the two-hybrid host

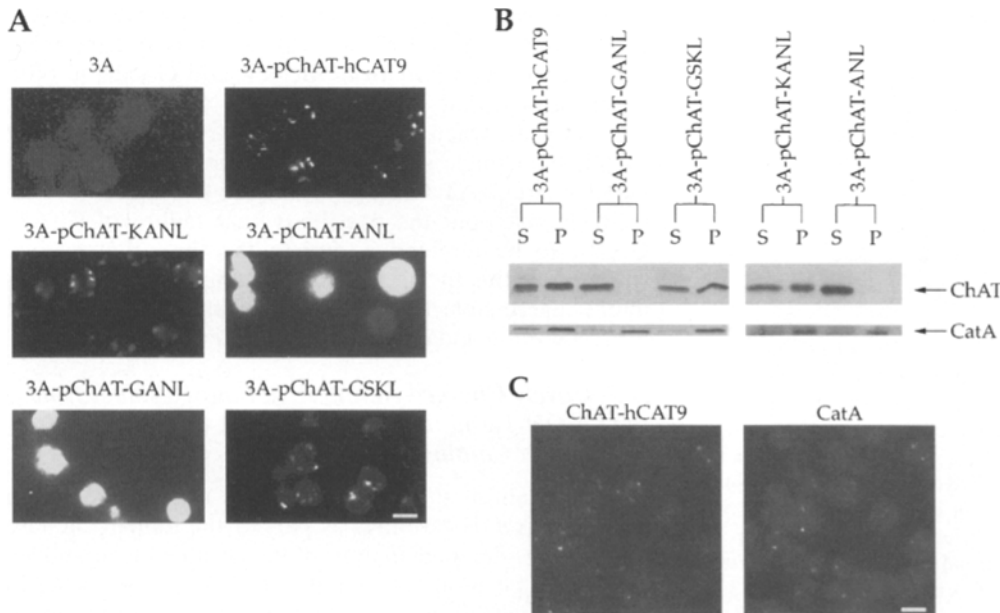


Figure 4. Distribution of transgenic chloramphenicol acetyl transferase (ChAT) variants in yeast cells, as assessed by immunofluorescence and immunoblotting. (A) 3A cells expressing ChAT appended at the COOH-terminus with either -KANL (3A-pChAT-KANL), -GANL (3A-pChAT-GANL), the last nine residues of human catalase (3A-pChAT-hCAT9), -ANL (3A-pChAT-ANL), or -GSKL (3A-pChAT-GSKL), were subjected to immunofluorescence with polyclonal anti-ChAT. 3A = untransformed negative control. Bar, 5 μ m. (B) Post-nuclear supernatant fractions were separated by differential centrifugation (61) into organellar pellets (P), and high speed supernatants (S), and subjected to SDS-PAGE and immuno-

blotting. Anti-ChAT was used to detect the various forms of transgenic ChAT (upper portion of figure), followed by anti-catalase A (lower portion of figure). (C). Colocalization of ChAT-hCAT9 and catalase A as revealed by double immunofluorescence of 3A-pChAT-hCAT9 with anti-ChAT and anti-catalase A. Bar, 5 μ m.

strain SFY526 with plasmids encoding the activation domain of GAL4 fused to Pas10p (pGAD424-pas10), and the DNA-binding domain of GAL4 fused to the COOH-terminal 146 amino acids of normal human catalase (pGBT9-hCAT1) resulted in colonies which synthesized β -galac-

tosidase (Fig. 6), indicating that Pas10p binds the COOH terminus of catalase. Controls lacking either Pas10p or human catalase did not synthesize β -galactosidase. Moreover, introduction of amino acid changes at either the penultimate residue (asparagine-to-aspartate), or four

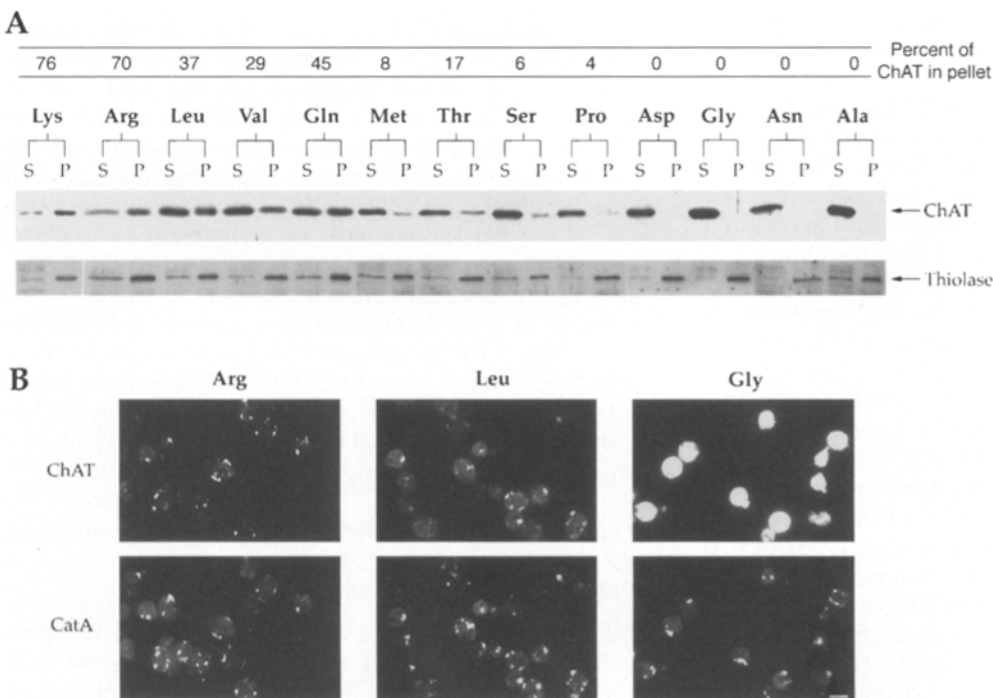


Figure 5. The fourth amino acid from the COOH terminus constitutes a critical component of the human catalase PTS, as assessed by immunoblotting and immunofluorescence. (A) 3A cells expressing various forms of ChAT-EXANL, where X is any of a number of different amino acids, were subjected to subcellular fractionation. Post-nuclear supernatant fractions were separated by differential centrifugation (61) into organellar pellets (P) and high speed supernatants (S), and subjected to SDS-PAGE and immunoblotting. Anti-ChAT was used to detect the various forms of transgenic ChAT (upper portion), followed by anti-peroxisomal thiolase (lower portion). Quantitative estimation of the proportion of ChAT in pellets (and supernatants) was performed using a Molecular Diagnostics Personal Densitometer. (B) Localization of various ChAT constructs and catalase A as revealed by double immunofluorescence of 3A-pChAT-ERANL (Arg), 3A-pChAT-ELANL (Leu), and 3A-pChAT-EGANL (Gly) with anti-ChAT (upper panels) and anti-catalase A (lower panels). Occasional ChAT-negative cells can be accounted for by plasmid loss during the YPGO growth of the cells. Bar, 5 μ m.

various ChAT constructs and catalase A as revealed by double immunofluorescence of 3A-pChAT-ERANL (Arg), 3A-pChAT-ELANL (Leu), and 3A-pChAT-EGANL (Gly) with anti-ChAT (upper panels) and anti-catalase A (lower panels). Occasional ChAT-negative cells can be accounted for by plasmid loss during the YPGO growth of the cells. Bar, 5 μ m.

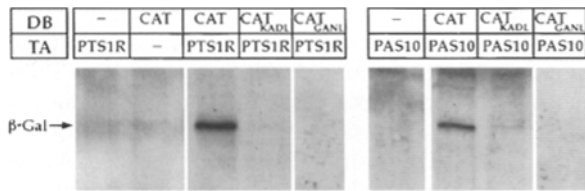


Figure 6. Interaction between the human catalase PTS and PTS1 receptors of yeast and humans in the yeast two-hybrid assay. β -galactosidase expression was determined by immunoblotting with anti- β -galactosidase, using 25 μ g aliquots of glass-bead homogenates. In each case yeast host strain SFY526 had been transformed with two plasmids. One plasmid contains the GAL4 DNA-binding domain (DB) fused to the last 146 amino acids of human catalase with normal COOH-terminal sequence (CAT) or with this sequence mutated to -KADL (CAT_{KADL}) or -GANL (CAT_{GANL}). The other plasmid contains the GAL4 transactivation domain (TA) fused to either the yeast *PAS10* gene (PAS10) or the cDNA encoding amino acids 238-639 of the human PTS1 receptor (PTS1R). -, vector alone, expressing unmodified GAL4 domains.

residues from the COOH terminus (lysine-to-glycine), both of which abolish the catalase PTS function, also eliminated the interaction with Pas10p in the two-hybrid assay (Fig. 6), suggesting that it is the PTS itself which is binding to Pas10p. To further test the role of Pas10p in the functioning of the human catalase PTS, ChAT-KANL and ChAT-hCAT9 were expressed in yeast strain 3A- Δ *pas10*, in which the *PAS10* gene had been disrupted. Immunofluorescence analysis revealed that ChAT-KANL (and ChAT-hCAT9) remain cytosolic in these cells, whereas thiolase (which is targeted by an NH₂-terminal PTS2 signal) is peroxisomal (Fig. 7). This contrasts with the peroxisomal location of ChAT-KANL (and ChAT-hCAT9) when expressed in wild-type cells (Fig. 4 A), and confirms that the human catalase COOH-terminal PTS does belong to the PTS1 family of peroxisomal targeting signals.

The Identified Human Catalase PTS Is Also Functional in Human Cells

To evaluate whether the COOH-terminal PTS of human catalase that is responsible for targeting in yeast also functions in human cells, the constructs encoding ChAT-GSKL, ChAT-GANL, ChAT-hCAT9, and ChAT-KANL were subcloned into a suitable vector containing the CMV promoter (see Materials and Methods), and transfected into human fibroblasts.

Immunofluorescence analysis of the transfected cells revealed a pattern of targeting in the human cells entirely equivalent to that found in yeast cells. As shown in Fig. 8, ChAT-GSKL, ChAT-KANL, and ChAT-hCAT9 were routed to peroxisomes, as witnessed by punctate fluorescence with anti-ChAT, whereas cells expressing ChAT-GANL exhibited fluorescence throughout the cytosol, consistent with failure to import this variant. To test the role of the human PTS1 receptor in the functioning of the last four amino acids of human catalase as a PTS, ChAT-KANL and ChAT-hCAT9 were expressed in fibroblasts of a neonatal adrenoleukodystrophy patient containing a missense mutation encoding an asparagine-to-lysine substitution at residue 489 of the PTS1 receptor. This mutation

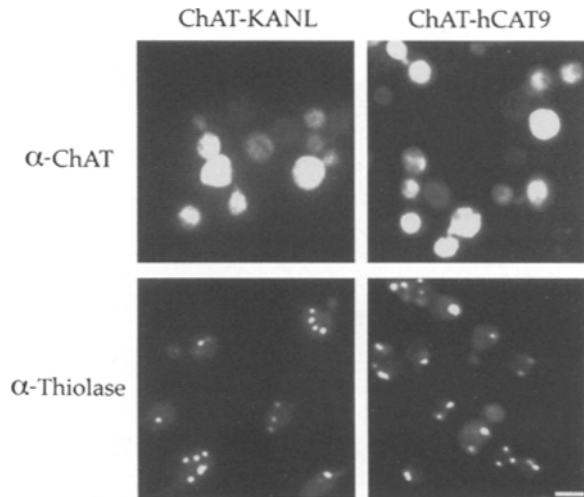


Figure 7. Dependence of human catalase PTS function upon the yeast PTS1 receptor, Pas10p. ChAT-KANL and ChAT-hCAT9 were expressed in 3A- Δ *pas10* cells, which lack Pas10p, and detected by immunofluorescence using anti-ChAT (upper panels). Peroxisomes were detected with anti-thiolase (lower panels). All of the fluorescent cells in the upper panels show cytosolic fluorescence when viewed while focusing up and down. Bar, 5 μ m.

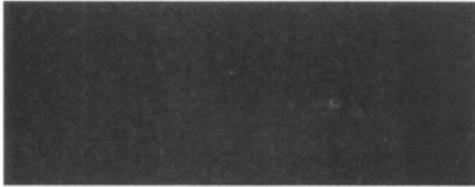
abolishes the import of proteins targeted by PTS1 signals, whereas PTS2-targeted proteins are imported normally (16). Immunofluorescence analysis of this cell line revealed a cytosolic distribution of endogenous catalase (data not shown), in agreement with previously published results (44). ChAT-KANL had a cytosolic distribution in these cells, as did ChAT-hCAT9 (Fig. 8). These data show that the human catalase PTS requires a functional PTS1 receptor, and confirm that the particles observed in wild-type fibroblasts (Fig. 8) are indeed peroxisomes. In addition, human catalase interacted with the human PTS1 receptor in a yeast two-hybrid assay (Fig. 6). Altering the penultimate residue of catalase from asparagine to aspartate abolished the interaction, as did changing lysine to glycine four residues from the COOH terminus. These results demonstrate that the human catalase PTS identified by the transgenic studies in yeast cells most probably represents the genuine PTS of this protein.

Peroxisomal Import of Fusion Proteins Between Yeast Catalase A and Human Catalase

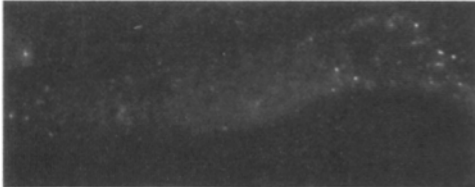
A recent investigation of the peroxisomal targeting of yeast catalase A revealed the presence of at least two independent PTSs. The last six residues of catalase A, -SSN-SKF, were shown to be sufficient for peroxisomal targeting of reporter proteins, but this hexapeptide was dispensable for catalase A targeting due to the existence of a second, internal PTS, tentatively identified as being located between residues 104-126 (30). The results presented above suggest that peroxisomal targeting of human catalase in yeast is similar to catalase A targeting in that a COOH-terminal PTS is present, but different in that loss of this PTS abolishes peroxisomal targeting. This is somewhat surprising given the sequence similarities between the two catalases in the region corresponding to the internal catalase A PTS (see Fig. 9). In the light of this, we investi-

Normal Fibroblasts

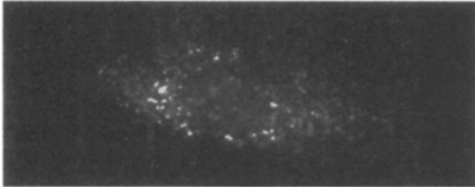
Mock (-DNA)



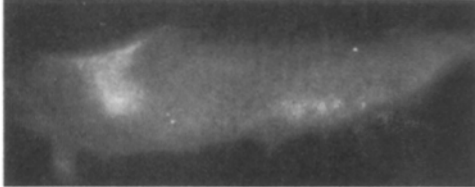
ChAT-hCAT9



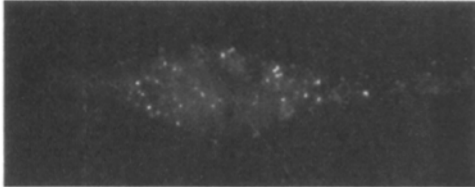
ChAT-KANL



ChAT-GANL

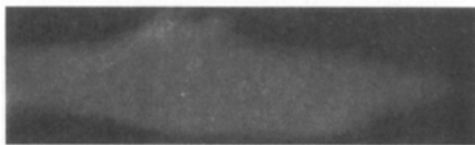


ChAT-GSKL



NALD Fibroblasts

ChAT-hCAT9



ChAT-KANL

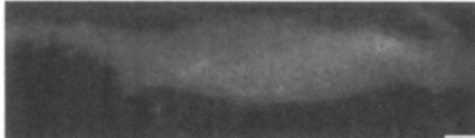


Figure 8. Human catalase PTS function in human cells. (*Top panels*) Peroxisomal targeting in normal human cells as assessed by immunofluorescence of normal human fibroblasts expressing ChAT-hCAT9, ChAT-KANL, ChAT-GANL, and ChAT-GSKL, and mock-transfected fibroblasts (no DNA, negative control). (*Bottom panel*) Dependence of human catalase PTS function upon the human PTS1 receptor. Neonatal adrenoleukodystrophy

gated the ability of yeast catalase A-human catalase fusion proteins to localize to peroxisomes, and the dependence of this on the COOH-terminal human catalase PTS. Plasmids encoding fusions consisting of the first 378 residues of catalase A followed by residues 384-527 of either normal human catalase (CATA-hCAT, encoded by pCATA-hCAT1), or catalase with the penultimate asparagine changed to aspartate (CATA-hCAT_{ADL}, encoded by pCATA-hCAT4) were constructed and transformed into 3A cells. These catalase fusions could be readily detected with antibody 10-330 by immunoblotting and immunoelectron microscopy, but not by immunofluorescence. Immunoelectron microscopic analysis demonstrated that the CATA-hCAT, which includes the intact human catalase COOH terminus, was located principally within the peroxisomal matrix and, interestingly, the labeling with gold particles was frequently confined to an electron dense sub-compartment of the organelle, suggesting that the catalase fusion may be forming into intraperoxisomal aggregates (Fig. 2 C).

Mutation of the COOH-terminal PTS of the fusion protein by changing the penultimate asparagine to aspartate (CATA-hCAT_{ADL}) did not abolish its peroxisomal targeting. Immunoelectron microscopy showed that this fusion was present in the peroxisomal matrix (Fig. 2 D). Consistent with this, cell fractionation revealed that CATA-hCAT_{ADL}, in contrast to hCAT_{ADL}, was found in the organellar pellet, although the efficiency of targeting appeared to be lower than for CATA-hCAT (see Fig. 3). Thus, the internal catalase A PTS is functional to some degree in this construct.

The possibility that peroxisomal targeting of the catalase A-human catalase fusions might involve interaction with the biogenesis pathway of endogenous catalase A was investigated by expression of these fusions in strain 3A- Δ CatA, which lacks catalase. Cell fractionation data revealed that this disruption had no discernible impact on the proportions of the various human catalase constructs found in the organellar pellets (Fig. 3).

Discussion

In this paper we provide evidence that targeting of human catalase to peroxisomes is mediated by a novel COOH-terminal PTS which consists of four amino acids. Deletion of these last four residues (KANL), or alteration of the penultimate asparagine to aspartate, abolished localization of human catalase to yeast peroxisomes. By analogy with other characterized PTSs, the COOH-terminal location of the human catalase PTS suggests that it is most likely a member of the class of COOH-terminal tripeptide PTSs collectively known as PTS1, and this is supported by evidence (discussed below) that this PTS cannot function in yeast or human cells in which the PTS1 receptor is absent or nonfunctional. However, this PTS differs from a classical PTS1 in two major respects.

(NALD) fibroblasts with a missense mutation in the PTS1 receptor expressing ChAT-hCAT9 and ChAT-KANL. The antibody was anti-ChAT. The very occasional bright spots in the case of ChAT-GANL appear to be on the cell surface. Bar, 10 μ m.

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1      104      126      515
|      |      |      |
MSKLG...CLTRFSTVGGDKGSADTVRDRPG.....SSNSKF      YEAST CATALASE A
|||||
MADSR...IAVRFSTVAGESGSADTVRDRPG.....SHLAAREKANL      HUMAN CATALASE
1      109      131      527

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Figure 9. Comparison of the sequences of human catalase and yeast catalase A. Regions shown to correspond to PTSs are underlined. Numbers refer to amino acids and relative to the amino terminus.

The first of these is the absence of a basic residue at the penultimate position. The PTS1 consensus sequence for mammals defines an absolute requirement for a basic residue at this position, with PTS functionality being abolished in every instance of alteration of the penultimate residue to a nonbasic amino acid, both in vitro (43) and in vivo (23). In *S. cerevisiae*, variants of this class of PTS previously shown to be active include SKL (multifunctional protein [28], peroxisomal citrate synthase [56], peroxisomal malate dehydrogenase [36, 58], peroxisomal malate synthase [27, 38], and transgenically expressed firefly luciferase [24]), SKF (catalase A [30]), AKL (mutant transgenic chloramphenicol acetyl transferase [40]), and AKI (transgenically expressed *Candida tropicalis* multifunctional protein [1]), all of which have lysine as the penultimate residue. Moreover, mutating the COOH-terminal AKI of *Candida tropicalis* multifunctional protein to -AQI abolished peroxisomal targeting of this protein in *S. cerevisiae*. Similarly, in *Hansenula polymorpha*, a basic penultimate residue is a constant feature of identified COOH-terminal PTS1 signals (15, 26, 47).

The second unusual feature of the human catalase PTS is that it is not restricted to the COOH-terminal three amino acids. This is shown by the failure of the tripeptide -ANL to function in place of -SKL in targeting a reporter protein, chloramphenicol acetyl transferase (ChAT), to human or yeast peroxisomes when appended to the COOH terminus via a single glycine residue (ChAT-GANL). This is not due to ChAT-GANL assuming a three-dimensional structure which renders the COOH terminus unavailable for interaction with the PTS1 recognition and import machinery, since McNew and Goodman (40) have shown that an equivalent construct with the archetypal PTS1, -SKL (ChAT-GSKL) is efficiently imported into *S. cerevisiae* peroxisomes. We have confirmed this observation in yeast cells and extended it to human cells. Moreover, McNew and Goodman (40) have shown that the PTS1 variant -AKL can function in this context (ChAT-GAKL) in yeast. Rather, failure of ChAT-GANL to localize to peroxisomes can more satisfactorily be explained as follows: the human catalase PTS is not restricted to the COOH-terminal tripeptide. This postulate is supported by the finding that ChAT can be directed to yeast peroxisomes when the last four residues of human catalase are appended to the COOH terminus (ChAT-KANL). This result indicates that the lysine residue four amino acids from the COOH terminus constitutes a critical component of the human catalase PTS, and this is borne out by the observation that substitution of this residue with a variety of different amino acids abolishes, or reduces, peroxisomal targeting. Significantly, the amino acid tested which resulted in little or no decrease in targeting

was arginine. This argues that the critical requirement at this position of the human catalase PTS for maximal targeting activity may be a basic residue. However, the fact that significant levels of targeting were also observed with nonbasic residues such as glutamine and leucine at this position suggests that the detailed structure-function relationship of this targeting sequence is more complex than an absolute requirement for a basic residue at the -4 position.

In the yeast *Pichia pastoris*, disruption of the *PAS8* gene, which encodes the PTS1 receptor (60), results in mistargeting of a range of peroxisomal proteins including transgenic luciferase (terminates -SKL), methanol oxidase (terminates ARF), and dihydroxyacetone synthase (terminates DKL) (39). The implication is that Pas8p is a PTS1 receptor with broad specificity, recognizing a variety of COOH-terminal tripeptides, but with a basic residue at the central position being a consistent feature. Homologues to *P. pastoris* *PAS8* have been identified in *S. cerevisiae* (64), *Hansenula polymorpha* (46, 63), and humans (16, 21, 68) suggesting that this mechanism of import is conserved. Against this body of evidence for a critical role of the penultimate basic residue of PTS1 signals, the only example of a nonbasic residue being tolerated at this position is AQI (at the COOH terminus of mutant transgenically expressed *C. tropicalis* multifunctional protein) in *Candida albicans* (1), other than targeting to trypanosomal glycosomes, which is uniquely supported by a tremendous range of COOH-terminal PTSs (6, 57). Therefore, the identification within human catalase, which ends -KANL, of a significantly new PTS1 variant with asparagine (as opposed to a basic amino acid) at the penultimate position, is both unexpected and notable.

That the human catalase COOH-terminal PTS sequence is a member of the PTS1 family is borne out by the observations that targeting by this sequence is abolished in yeast cells in which the PTS1 receptor (Pas10p) is absent, and in human cells in which the PTS1 receptor is mutated. Furthermore, use of the two-hybrid system has shown that both the yeast Pas10p and human PTS1 receptor can interact with the COOH-terminal sequence of normal catalase (ends -KANL), but not with mutated forms (ending -KADL or -GANL) which are nonfunctional as PTSs. It remains to be seen whether any naturally occurring peroxisomal proteins in this yeast contain a PTS1 signal with asparagine at the penultimate position. The fact that antibodies against a synthetic peptide terminating with AKI show only a very weak reaction with a single *S. cerevisiae* peroxisomal protein, although this tripeptide is active as a PTS1 in this yeast (2), suggests that the range of PTS1s used may be more limited than the full spectrum of permissible tripeptides identified by mutagenesis and heterologous expression studies.

Inclusion of human catalase as a PTS1 targeted protein raises something of a conundrum, namely that if a basic penultimate residue is not essential, why have not variants lacking this feature been previously identified among the wide range of characterized PTS1 signals? One intriguing possibility is that the residue (lysine) immediately adjacent to the -ANL terminus which, as described above, forms part of the PTS, is critically involved in compensating for the lack of a basic penultimate amino acid. Significantly,

an analogous scenario has recently been proposed for another unconventional mammalian PTS1 variant, namely the COOH-terminal -KKL signal of human alanine:glyoxylate aminotransferase (AGT) (45). Like the -ANL terminus of catalase, this tripeptide differs from the mammalian PTS1 consensus at one of the three residues, the deviation in this case being a lysine residue three amino acids from the COOH terminus, as opposed to serine or alanine. Also in common with the catalase tripeptide is the observation that the -KKL is necessary for peroxisomal targeting (of AGT), but is not sufficient to functionally replace -SKL as a PTS for chloramphenicol acetyl transferase. This has led to the suggestion that some other region of AGT is required for functional expression of the COOH-terminal targeting sequence (45). Thus, although it must be borne in mind that this proposed ancillary sequence of AGT remains ill-defined, these results appear to suggest that targeting of AGT, like catalase, may involve additional amino acids which somehow compensate for the existence of nonconsensus COOH-terminal PTS1 signals. Likewise, it may be that more conventional PTS1 signals are similarly modulated by adjacent sequences. For instance, in vitro peroxisomal import of a truncated (non-import competent) form of catalase is supported much more efficiently by addition of the last five amino acids of rat acyl-CoA oxidase (-LQSKL) than by -SKL alone, and inhibition of in vitro peroxisomal import of rat acyl-CoA oxidase with synthetic peptides has been shown to be much more effective with a peptide corresponding to the last 10 residues of rat acyl-CoA oxidase (-KHLKPQSKL) than with an -SKL tripeptide (43). In addition, chloramphenicol acetyl transferase appended with -AKI has been mentioned as failing to localize to peroxisomes, despite evidence that -AKI can function as a yeast PTS1 (1).

Alteration of the internal -SHL- tripeptide near the COOH terminus of human catalase to -TQV- did not prevent targeting to yeast peroxisomes, effectively ruling out this tripeptide as a novel "internal PTS1" targeting sequence. Interest in this tripeptide was aroused by the demonstration that the last 27 residues of human catalase were sufficient for targeting to peroxisomes in mammalian cells despite the fact that the terminal -ANL sequence is outside the defined consensus for mammalian PTS1 signals, whereas SHL is within this consensus (23, 25). As such, this internal SHL tripeptide has represented the most likely of a number of internal tripeptides speculated to function in peroxisomal targeting. The observations that an addition of a single serine residue to the COOH terminus of luciferase abolished its peroxisomal localization in mammalian cells (23), and that inhibition of in vitro peroxisome import with synthetic SKL-containing peptides is only observed if the SKL is at the extreme COOH terminus of the peptide (43), had indicated that COOH-terminal PTSs were rendered inactive when moved from the extreme COOH terminus. In addition, it has recently been demonstrated that an internal SKL tripeptide is not involved in the targeting of the peroxisomal membrane protein PMP47 in *Candida boidinii* (37). Combined with these observations, our finding that the internal SHL of human catalase is not a PTS now suggests that it is unlikely that the PTS1 family of targeting sequences will include internal tripeptides.

Comparison of the results of the mutagenesis of the extreme COOH terminus of human catalase with similar experiments using yeast catalase A reveals both similarities and differences. Like human catalase, catalase A appears to have a COOH-terminal PTS1 (-SSNSKF) (30). However, deletion of this signal does not prevent targeting of catalase A to peroxisomes, due to the presence of a second, independent targeting sequence internal to the protein. Clearly, this second PTS is not conserved (in terms of functionality in yeast) in the human protein, despite the fact that a preliminary delineation has identified the internal PTS as being between residues 104-126 of catalase A (30), a region which shows substantial conservation between yeast and humans. Consistent with these data, we have shown that hybrid catalases, of which approximately the NH₂-terminal three-quarters is derived from yeast catalase A, and the remainder from human catalase, can be targeted to yeast peroxisomes whether the human COOH-terminal PTS is intact (-ANL) or disrupted (-ADL).

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