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, human 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 generated 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...
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 determining the situation 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), 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 at the COOH-terminal 27 residues of human catalase (25). These 27 amino acids are of special interest in that they terminate with a sequence (-SHL-) which, nine to eleven residues from the COOH terminus, 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), implicating 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'-GGCAAGGGAGTAGGCAAAATC-3'; C3, 5'-TCCGGAATCGAGTTGGCCGCA-3'; C4, 5'-GAGAAGGCAGATC2AGGTGGCCGCCCGCCCTG-3'; C5, 5'-CGCAAGGAGGAGGCAGGGGCAAATCTGTGA-3'; A1, 5'-AAGGATCTTGGTCCATTTAGTGCAC-3'; A2, 5'-GGAATTCGGATACTCAG-3'; A3, 5'-CTTGGGAGAATTTCGAGAC-3'; CH2, 5'-CTAGAGATTGCCGCTTCTCCGTCTGCAGCGCCGCGCCCGCATGCTGCT-3'; CH3, 5'-CTAGAGATTGCCGCTTCTCCGTCTGCAGCGCCGCGCCCGCATGCTGCT-3'; CH4, 5'-CTAGAGATTGCCGCTTCTCCGTCTGCAGCGCCGCGCCCGCATGCTGCT-3'; CH5, 5'-CTAGAGATTGCCGCTTCTCCGTCTGCAGCGCCGCGCCCGCATGCTGCT-3'; CH6, 5'-CTAGAGATTGCCGCTTCTCCGTCTGCAGCGCCGCGCCCGCATGCTGCT-3'; CH7, 5'-CTAGAGATTGCCGCTTCTCCGTCTGCAGCGCCGCGCCCGCATGCTGCT-3'; CH8, 5'-CTAGAGATTGCCGCTTCTCCGTCTGCAGCGCCGCGCCCGCATGCTGCT-3'.

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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 uM dNTPs, and 2 uM of each primer. Samples were heated to 95°C for 5 min before addition of 2.5 uL of Taq Polymerase (Boehringer Mannheim Corp., Mannheim, FRG). Thirty five cycles of 95°C (90 s), 55°C (60 s), 72°C (20 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.
Construction of Plasmids

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

phCAT1 (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 pUC18, 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 phCAT1 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 Accl–XhoI fragment, which was then used to replace the CiaI–XhoI fragment (which replaces 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 Smal site of pT7Blue, and then subcloning (using SallA) into pPOX1 (which contains the promoter of yeast acetyl CoA 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 products, respectively. For the construction of 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 pCMV-Link, a vector constructed by the insertion of the pBluescript KS(+) polylinker into pCMVII (35), as replacement of the pBluescriptKS (+) nonapeptide replaced with an additional nonapeptide corresponding to the human catalase COOH terminus, under control of the promoter of the S. cerevisiae 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

For catalase A disruption.

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).

pGVT9-hCAT1 GAL4 DNA-binding domain fused to residues 382-527 of human catalase, under control of ADH promoter.

pGVT9-hCAT4 pGVT9-hCAT1, but with COOH-terminus of fusion protein mutated to -KADL
pGVT9-hCAT1KG pGVT9-hCAT1, but with COOH-terminus of fusion protein mutated to -GANL
pCATA-hU3A URA3 gene cloned within catalase A coding region. For catalase A disruption.

Yeast Strains

Our laboratory "wild-type" strain 3A (MATa, ura3-1, trpl-1, arg4, cat1-1) (JW68-3A in [72]) is deficient in cytosolic catalase T, but does contain peroxisomal catalase A. Strain 3A-DcatA (MATa, ura3-1, trpl-1, arg4, cat1-1, ...

Table I. Plasmids Used in This Study

| Name         | Description                                           |
|--------------|-------------------------------------------------------|
| pCAT10       | Full-length human catalase cDNA cloned into EcoRI site of pSP64. |
| pCATmut1     | Complete human catalase cDNA coding sequence cloned into pSELECT |
| pCAT1        | Complete human catalase cDNA coding sequence cloned into pCUP1 |
| pCAT2        | phCAT1, mutated to encode catalase lacking the COOH-terminal 4 residues |
| pCAT3        | phCAT1, mutated to encode catalase with internal -SHL- changed to -TQH- |
| pCAT4        | 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 S. cerevisiae catalase A gene encoding residues 1-378 |
| pCATA-hCAT4  | phCAT4, with region encoding human catalase residues 1-383 replaced with the portion of S. cerevisiae 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 S. cerevisiae 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 pCMV8 |
| 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) |
| pGVT9-hCAT1  | GAL4 DNA-binding domain fused to residues 382-527 of human catalase, under control of ADH promoter |
| pGVT9-hCAT4  | pGVT9-hCAT1, but with COOH-terminus of fusion protein mutated to -KADL |
| pGVT9-hCAT1KG | pGVT9-hCAT1, but with COOH-terminus of fusion protein mutated to -GANL |
| pCATA-hU3A   | URA3 gene cloned within catalase A coding region. For catalase A disruption |

Purdue and Lazarow Catalase Peroxisomal Targeting Sequence
ga180-538, 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 (MATa, ura3-1, ade2-101, his3-200, 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-Δpas10 (MATa, 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, 10⁵ cells were trypsinized with 0.025% trypsin in phosphate-buffered saline (PBS), and seeded at a 1:5 dilution into 60-mm petri dishes. After subconfluence 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 µg/ml Zymolyase and 3 µM 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 Vienna, Wien, Austria) were used at 1:100-1:2000. 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 conjugated 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 dye-exchange 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 14,000 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 pCATA10, PAC1002, pF3SF1-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 Fransen (University of Leuven), and Dr. Jing-Wei Zhang (Mount Sinai School of Medicine, New York), respectively. Restriction nuclease 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 organelle perinl 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 (hCATa4; 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 μm.

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_{a4}) 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 organelar 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 (hCATADL); (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-hCATADL). 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 μm.
Figure 3. Immunoblot analysis of subcellular localization of various forms of catalase. 3A and 3A-\(\Delta\)CatA were transformed with plasmids encoding normal human catalase (hCAT; plasmid pHCAT1), human catalase with the penultimate residue changed from asparagine to aspartate (hCATADL; plasmid pHCAT4), a fusion protein consisting of residues 1-378 of yeast catalase A fused to residues 384-527 of normal human catalase (CATA-hCAT1; plasmid pCATA-hCAT1), and an identical fusion protein to CATA-hCAT, but with the penultimate residue changed from asparagine to aspartate (CATA-hCATADL; plasmid pCATA-hCAT4). Postnuclear supernatant fractions were separated by differential centrifugation (61) into organelar pellets (P) and high speed supernatants (S), which were then subjected to SDS-PAGE and immunoblotting. Antibody 10-330 (\(\alpha\)-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 (\(\alpha\)-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 organelar 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 (\(\alpha\)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, \(-\text{SKL}\) (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 \(-\text{SKL}\) and \(-\text{AKL}\) 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, asparagline, 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 organelar 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 \(-\text{SKL}\) 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-CAT9), -ANL (3A-pChat-ANL), or -GSKL (3A-pChat-GSKL), were subjected to immunofluorescence with polyclonal anti-Chat. 3A = untransformed negative control. Bar, 5 μm. (B) Postnuclear 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 of figure), followed by anti-catalase A (lower portion of figure). (C) Colocalization of Chat-CAT9 and catalase A as revealed by double immunofluorescence of 3A-pChat-CAT9 with anti-Chat and anti-catalase A. Bar, 5 μm.

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-galactosidase (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...
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-Apas10, 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 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 asparagine abolishes 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-
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 

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.
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 PTSs 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. Again 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 (ends -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 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,
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 (SSNKSFK) (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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