Human Peroxisomal Targeting Signal-1 Receptor Restores Peroxisomal Protein Import in Cells from Patients with Fatal Peroxisomal Disorders

Erik A. C. Wiemer,* William M. Nuttley,* Bonnie L. Bertolaet,† Xu Li,§ Uta Francke,¶ Margaret J. Wheelock,‖ Usha K. Annr,‖ Keith R. Johnson,‖ and Suresh Subramani*†
†Department of Biology, University of California at San Diego, La Jolla, California 92093-0322;‡The UCSD Cancer Center, University of California at San Diego, La Jolla, California 92093-0636;§Howard Hughes Medical Institute, Departments of Genetics and Pediatrics, Stanford University Medical Center, Stanford, California 94305-5428; and ‖Department of Biology, University of Toledo, Toledo, Ohio 43606-3390

Abstract. Two peroxisomal targeting signals, PTS1 and PTS2, are involved in the import of proteins into the peroxisome matrix. Human patients with fatal generalized peroxisomal deficiency disorders fall into at least nine genetic complementation groups. Cells from many of these patients are deficient in the import of PTS1-containing proteins, but the causes of the protein-import defect in these patients are unknown. We have cloned and sequenced the human cDNA homologue (PTS1R) of the \textit{Pichiapastoris PAS8} gene, the PTS1 receptor (McCollum, D., E. Monosov, and S. Subramani. 1993. \textit{J. Cell Biol.} 121:761–774). The PTS1R mRNA is expressed in all human tissues examined. Antibodies to the human PTS1R recognize this protein in human, monkey, rat, and hamster cells. The protein is localized mainly in the cytosol but is also found to be associated with peroxisomes. Part of the peroxisomal PTS1R protein is tightly bound to the peroxisomal membrane. Antibodies to PTS1R inhibit peroxisomal protein-import of PTS1-containing proteins in a permeabilized CHO cell system. In vitro–translated PTS1R protein specifically binds a serine-lysine-leucine–peptide. A \textit{PAS8–PTS1R} fusion protein complements the \textit{P. pastoris pas8} mutant. The PTS1R cDNA also complements the PTS1 protein-import defect in skin fibroblasts from patients—belonging to complementation group two—diagnosed as having neonatal adrenoleukodystrophy or Zellweger syndrome. The PTS1R gene has been localized to a chromosomal location where no other peroxisomal disorder genes are known to map. Our findings represent the only case in which the molecular basis of the protein-import deficiency in human peroxisomal disorders is understood.

Transport of proteins into the peroxisomal matrix occurs via at least two pathways dependent on distinct peroxisomal targeting signals (PTS1 and PTS2). The PTS1 sequence is a COOH-terminal tripeptide (serine-lysine-leucine [SKL] or a variant) (Gould et al., 1989), while the PTS2 sequence is an NH$_2$-terminal peptide (Swinkels et al., 1991; Osumi et al., 1991). Both sequences are necessary and sufficient for peroxisomal targeting and are used by evolutionarily diverse organisms (Subramani, 1993).

Peroxisomes are intimately involved with many important biochemical pathways (Van den Bosch et al., 1992; Mannerts and Van Veldhoven, 1993) and a variety of diseases in humans (Lazarow and Moser, 1989; Wanders et al., 1988). One class of these disorders (group A) is characterized by a generalized loss of peroxisomal functions. Diseases belonging to this class include the cerebro-hepato-renal (Zellweger) syndrome (ZS), hyperpipericolic acidemia (HPA), the neonatal form of adrenoleukodystrophy (nALD) and infantile Refsum’s disease. Patients suffering from ZS are clinically characterized by severe craniofacial dysmorphism, hypotonia, seizures, hepatomegaly, renal cysts, and adrenal atrophy. They often die within a year after birth. nALD, HPA, and infantile Refsum’s disease are also lethal disorders, but present themselves more mildly, resulting in a somewhat longer life span (Wanders et al., 1988).
Cells from patients with these disorders have been placed into nine complementation groups (see Shimozawa et al., 1993 for an overview). All cells contain peroxisome ghosts (Santos et al., 1988a,b; Wiemer et al., 1989). At least six complementation groups exhibit a deficiency in the import of PTS1-containing proteins (Walton et al., 1992; Wendland and Subramani, 1993b; Motley et al., 1994). The genes affected in two of the nine complementation groups have been identified, and encode integral peroxisomal membrane proteins (PMPs) (Gartner et al., 1992; Shimozawa et al., 1992), but the molecular basis of the protein-import defect in these cells remains unexplained.

Both genetic and biochemical evidence exists for the PTS1 and PTS2 pathways. The pas8 mutant of Pichia pastoris (McCullom et al., 1993), the pas10 mutant of Saccharomyces cerevisiae (Van der Leij et al., 1993) and fibroblasts from a patient with neonatal adrenoleukodystrophy (Motley et al., 1994) are selectively deficient in the PTS1-pathway, but not the PTS2-pathway of import. Conversely, the pas7 mutant of S. cerevisiae (Kunau and Hartig, 1992; Marzoichi et al., 1994) and patients suffering from the rhizomelic form of chondrodysplasia punctata (Motley et al., 1994) are deficient only in the PTS2-pathway of import.

Cloning and characterization of the P. pastoris PAS8 gene reveal that the protein it encodes is the PTS1 receptor (McCullom et al., 1993; Terlecky et al., 1995). Because of its central role in the import of PTS1-containing proteins, and the impairment of this pathway of import in many of the group A peroxisomal disorders, we undertook the cloning of the human homologue (PTS1R) of the P. pastoris PAS8 gene. We describe the role of the human PTS1R protein in the PTS1-pathway of peroxisomal protein import, and in correcting this protein import defect in two fatal peroxisomal disorders (ZS and nALD).

Materials and Methods

Strains, Cell Lines, and Culture Conditions

Transformation and culture conditions of the methylotrophic yeast Pichia pastoris were essentially performed as described by Gould et al. (1992). Standard rich medium for growth of P. pastoris was YPD medium (1% yeast extract, 2% bactopectone, 2% glucose). Defined synthetic medium was prepared in pBSKSII (Stratagene, Inc. La Jolla, CA) creating pBS-PTS1R. Both strands were sequenced. A full-length PTS1R construct was prepared in pBSKSII (Stratagene, Inc. La Jolla, CA) creating pBS-PTS1R.

Liver homogenates were prepared as described by Krisans et al. (1994). Liver homogenates were prepared as described by Krisans et al. (1994). Liver homogenates were prepared as described by Krisans et al. (1994). Liver homogenates were prepared as described by Krisans et al. (1994). Liver homogenates were prepared as described by Krisans et al. (1994). Liver homogenates were prepared as described by Krisans et al. (1994).

Autopsy Material

Liver samples from two patients with ZS (unknown complementation group) and from two controls were kindly supplied by Dr. Hugo Moser.

Antibodies

Anti-PTS1R antibodies were raised as follows: a HindIII-partial BamHI (nucleotides 663–1847) fragment was cloned into the filled-in EcoRI site of the pGEX-KG polylinker (Guan and Dixon, 1991). The glutathione-S-transferase (GST)-PTS1R fusion protein was overexpressed in Escherichia coli DH5α as described by Guan and Dixon (1991) except that no benzamidin was used and the cells were lysed by sonication. The fusion protein proved to be insoluble and mostly was recovered in the pellet after the bacterial lysate was centrifuged at 10,000 g for 10 min at 4°C. The insoluble fraction was dissolved in protein sample buffer and subjected to SDS-PAGE. The GST-PTS1R fusion protein was electroeluted from the gel and dialyzed against PBS and subsequently thrombin buffer (50 mM Tris-CI, pH 8.0, 150 mM NaCl; 2.5 mM CaCl2; and 0.1% [vol/vol] β-mercaptoethanol) for 24 h at room temperature. The GST part of the fusion protein was cleaved off by a thrombin digest. The proteolytic fragments were separated on SDS-PAGE. The 45-kD PTS1R fragment was electroeluted and used to immunize a rabbit.

A rabbit polyclonal antiserum against rat liver 3-ketoacyl-CoA thiolase and a guinea pig polyclonal anti-SKL serum were a gift from Dr. R. Rachubinski (University of Alberta, Edmonton, Alberta, Canada). Anti–PMP70 was prepared in a rabbit and guinea pig using a PMP70 peptide conjugated to human serum albumin (HSA) as described by Kamijo et al. (1990). A rabbit antiserum raised against an SKL-peptide was prepared as described by Gould et al. (1990). A polyclonal rabbit antiserum was generated against bovine catalase. Polyclonal rabbit and guinea pig antinera directed against HSA were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Species-specific anti-lg antibodies conjugated to FITC or rhodamine were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cloning and Sequencing of the PTS1R Gene

A partial cDNA encoding PTS1R (nucleotides 441–3075) was inadvertently isolated while screening a human liver cDNA expression library in λgt11 (Clontech Laboratories, Inc., Palo Alto, CA) for cell adhesion-related proteins using a monoclonal antibody against β-catenin. To facilitate DNA sequencing, a set of nested deletions was generated by exonuclease III. Both strands of the resulting overlapping clones were sequenced. The 5' end of the cDNA was cloned by reverse transcription-PCR (RT-PCR), essentially according to Frohman (1990). A Rabies virus penta-gmo polylinker sites of the mammalian expression plasmid pM710 under the control of the cytomegalovirus promoter (Morgenstern and Land, 1990). The resulting pM710-PTS1R was used in the nuclear microinjection and transfection experiments. A HindIII-BglII (nucleotides 663–2050) fragment was cloned into frame into the filled-in EcoRI site of the T7 RNA polymerase/promoter plasmid pT7-7 (S. Tabor, Harvard Medical School, Boston, MA) thus creating pT7-7-PTS1R. In vitro transcription/translation of this construct produces a truncated PTS1R missing the NHE-terminus 221 amino acids.

A PAS8-PTS1R fusion construct was assembled in pSG464 (Gould et al., 1992) under control of the P. pastoris PAS8 promoter. The fusion pro-
tein consisted of the NH2-terminal 287 amino acids from PAS8 and the COOH-terminal 306 amino acids of PTS1R.

**Northern Blot Analysis**

A Northern blot, containing poly(A)+ RNA from multiple human tissues, was purchased from Clontech Laboratories, Inc. and used according to the recommendations of the manufacturer. A HindIII-Xhol (nucleotides 663-1534) fragment from PTS1R was uniformly labeled with [γ-32P]CTP and used as a probe. Subsequently the blot was stripped and reincubated with a radioactively labeled human β-actin cDNA probe.

**PAGE, Immunoblotting, Peroxisomal Membrane Preparations, Protein Concentration Measurements**

PAGE in the presence of SDS was carried out essentially as described by Laemmli (1970) and Western blotting to nitrocellulose filters according to Towbin et al. (1979). Remaining protein binding sites were saturated by incubating the filters for 1 h in PBS pH 7.4 containing 0.05% (vol/vol) Tween 20 (PBS-Tween) and 5% (wt/vol) low-fat milk powder. Subsequently the filters were incubated with antisera diluted in the same solution. Bound antibody was detected by incubation with a 1:5,000 dilution of goat anti-rabbit Ig conjugated to alkaline phosphatase (Bio-Rad Laboratories, Richmond, CA).

**Cell Fractionation and Isolation of Rat Liver Peroxisomes**

The experiments were performed with male Sprague-Dawley rats (180-240 g) that were fed a standard diet. Rats were fasted for 20 h before being killed. Liver homogenates were prepared and fractionated by differential centrifugation as described by Keller et al. (1986). A rat liver "light mitochondrial" fraction (λ-fraction) enriched in peroxisomes was further fractionated on a linear Nycodenz (Nycomed Pharma AS, Oslo, Norway) gradient as described by Keller et al. (1986). The characterization of the various gradient fractions for the activity of organellar marker enzymes, protein content, and the computer analysis of the data were performed essentially as described by Keller et al. (1986).

**In Vitro Import in Semi-permeabilized CHO Cells**

An in vitro import assay was performed using streptolysin-O (0.2 U/ml) permeabilized CHO cells and 50 μg/ml HSA-SKEL as a substrate (Wendland and Subramani, 1992a). Import was assessed by immunofluorescence microscopy with polyclonal antisera. HSA was detected using a rabbit anti-HSA serum at a 1:250 dilution. For experiments involving inhibition of import, 60-μl reactions were supplemented with 5 μl of anti-PTS1R serum or preimmune serum. IgG fractions were purified from PTS1R serum or preimmune serum. IgG fractions were purified from PTS1R serum or preimmune serum. IgG fractions were purified from PTS1R serum or preimmune serum.

**Fluorescence In Situ Hybridization (FISH)**

FISH on chromosomes was performed as described (Milatovich et al., 1991). Briefly, a cDNA clone containing a 2.4-kb insert of the human PTS1 receptor gene in pUC18 was labeled with biotin-16-dUTP by nick-translation using commercial reagents (Boehringer Mannheim, Mannheim, Germany). Labeled DNA probe was hybridized at a concentration of 400 ng/μl per slide to pretreated and denatured metaphase chromosomes from a human peripheral blood lymphocyte culture in the presence of sheared unlabelled salmon sperm DNA and human placental DNA as competitors. The slides were incubated at 37°C overnight and then washed and reacted with avidin/FITC (Vector Laboratories, Inc., Burlingame, CA). Hybridization signals were amplified by treatment with biotinylated goat anti-avidin D antibody (Vector Laboratories, Inc.) followed by another round of incubation with avidin/FITC. Chromosomes were counterstained with propidium iodide. At least 20 metaphase chromosomes were analyzed under an epifluorescence microscope (Axiophot, Carl Zeiss, Inc., Thornwood, NY), and images were collected with a CCD camera as described (Li et al., 1994). Hybridization signals were considered as specific only when the fluorescent signal was observed on both chromatids of a chromosome.

**Microinjection**

ALA-T, FAIR-T, and GM4340-T cells were microinjected with purified plasmid DNA (15-150 copies/cell) using a semiautomatic microinjector and manipulator (Eppendorf North America, Inc., Madison, WI) with an inverted microscope (Carl Zeiss, Inc.). Each expression vector (0.01 mg/ml) was injected directly into the nuclei of at least 200 cells along with guinea pig IgG (10 mg/ml) marker antibody in 100 mM KCl, 5 mM Na2PO4, pH 7.4, 6 h after injection, cells were fixed in 3.7% formaldehyde and further processed for immunofluorescence essentially as described by Keller et al. (1987) except that the fixed cells were permeabilized by a 0.3% (vol/vol) Triton X-100 solution in PBS for 15 min after which the cells were washed four times with PBS and incubated with the appropriate antiserum dilutions made up in PBS containing 0.5% (vol/vol) NP-40 and 5 mg/ml BSA. The subcellular localization of SKL-containing proteins was examined, by a first step incubation with a rabbit anti-SKL antiserum followed by a second incubation with an anti-rabbit Ig rhodamine. Injected cells were identified by anti-guinea pig Ig FITC.

**Mammalian Transfection**

ALA-T and FAIR-T cells were grown on glass coverslips and transfected

Wiener et al. *Human PTS1 Receptor Complements Peroxisomal Disorders*
Results

The Human PTS1R Is Homologous to the Yeast PTS1 Receptor

We cloned and sequenced a human cDNA that encodes a protein of 602 amino acids with an estimated molecular mass of 67 kD and isoelectric point of 4.2. The sequence (EMBL accession number Z48054) is likely to represent the full-length cDNA. When the 5' end was cloned by R-PCR, several PCR fragments were cloned that differed in length at their 5' ends. In the shorter clones that ended at positions −64 and −83, a G was found directly following the anchor primer. We believe these G's, which were absent in longer clones extending to −85, are derived from the cap structure of the full-length mRNA (Hirzmann et al., 1993). In extensive attempts to clone additional sequence at the 5' end, using reverse transcriptase-PCR with a set of nested oligonucleotides hybridizing to the extreme 5' end of the sequence, no longer clones were obtained. The first ATG encountered in the sequence (position 1–3) is most likely used as the start codon. Together with its flanking sequences it fits the consensus for an optimal translation start site (Kozak, 1984). Translation of the putative full-length cDNA in vitro produces a protein that migrates at a similar molecular weight in a SDS-polyacrylamide gel as the PTS1R protein in rat liver homogenates identified by a specific antiserum against PTS1R (Fig. 1). Finally, an expression construct starting with this ATG is most likely used as the start codon. Together with its flanking sequences it fits the consensus for an optimal translation start site (Kozak, 1984). Translation of the putative full-length cDNA in vitro produces a protein that migrates at a similar molecular weight in a SDS-polyacrylamide gel as the PTS1R protein in rat liver homogenates identified by a specific antiserum against PTS1R (Fig. 1). Finally, an expression construct starting with this ATG was able to complement the primary defect in group two peroxisomal disorders (see below).

The amino acid sequence defines the PTS1R protein as a member of the tetra peptide repeat (TPR) protein family to which proteins with diverse functions (chromosome segregation, cell cycle control, mitochondrial import, and transcription) belong (Goebi and Yanagida, 1991). A database search using the BLAST program and the PTS1R protein sequence as query detected a strong similarity with the P. pastoris PAS8 and S. cerevisiae PAS10 proteins. A number of other proteins belonging to the TPR protein family like nuc2+, BimA, CDC16, CDC23, STI1, and SSN6 were also found, but their similarity with the TPR domain of PTS1R was rather limited and fragmentary.

Alignment of the PTS1R primary amino acid sequence with the P. pastoris PAS8 protein and its homolog the S. cerevisiae PAS10 protein reveals the high degree of similarity (55.1 and 50.5%, respectively) with both proteins (Fig. 2). The overall identity between PTS1R versus PpPAS8 and ScPAS10 is 33.9 and 27%, respectively. The overall similarity and identity are not much higher when only PpPAS8 and ScPAS10 are compared (61.8 and 41%, respectively). The similarity is most striking in the TPR domain of the proteins and less so in their NH2-terminal parts. Not all TPR repeats are equally well conserved, in particular repeats three and four, corresponding to amino acid residues 366–450, show poor similarity. It is most likely that PTS1R, PpPAS8, and ScPAS10 are homologues.

Since it was demonstrated that the TPR domain of P. pastoris PAS8 binds the PTS1 (SKL) peptide (Terlecky et al., 1995) the ability of a PAS–PTS1R fusion protein (the NH2-terminal part of PAS8p linked to the TPR domain of PTS1R) to complement the P. pastoris pas8-I mutant provides a stringent test of PTS1R function in yeast. Table I shows that a differential complementation was observed, i.e., expression of the PAS8-PTS1R fusion restored the ability of the pas8-I mutant to grow on media containing oleate as sole C-source, however, it did not restore the ability to metabolize methanol.

PTS1R Is Expressed in Various Human Tissues

A single hybridizing band of ~3.4 kb, similar to the cDNA length, was observed when a Northern blot containing poly(A+) mRNAs from several human tissues was hybridized with a PTS1R probe (Fig. 3). The lower panel of Fig. 3 shows the same blot which, after stripping, was reprobed with a human β-actin cDNA probe. A variability in the level of PTS1R expression can be observed, however it cannot be unequivocally determined whether these variations are significant or are caused by the different amounts of RNA loaded onto the blot.

Assignment of PTS1R Gene to Chromosome Band 12p13.3

With primers complementary to 3' untranslated region sequences of the PTS1R gene the expected 541-bp PCR product was amplified from total human genomic DNA but not from hamster DNA. The specific PCR product was also obtained from human × Chinese hamster hybrid cell lines that had retained human chromosome 12. All other human chromosomes were excluded by the presence of at least three discordant hybrid cell lines (Table II). Furthermore, two hybrid cell lines retaining different partially deleted copies of chromosome 12 were used for regional mapping. Positive PCR amplification was observed in a hybrid cell line containing region 12pter-q21 but not in one containing only the long arm, region 12cen-qter.
These results indicate that the PTS1R gene is located on the short arm of chromosome 12 (Fig. 4).

Fluorescence in situ hybridization (FISH) using a PTS1R cDNA clone as probe independently confirmed the result from the hybrid mapping panel. Of 21 metaphase spreads analyzed, 16 exhibited a specific fluorescent signal at band p13.3 on both chromosomes of a chromosome 12, and in 9 of these cells signals were found on both chromosome 12 homologues. The chromosomes were identified based on an R banding pattern produced by the incorporation of bromodeoxyuridine after synchronization of the cells (Fig. 4).

**PTS1R Expression in Tissues and Mammalian Cell Lines**

An immunoblot, containing total protein fractions from...
Table I. Partial Complementation of Pichia pastoris pas8 Mutant by PAS8-PTS1R Fusion Construct

| Construct       | SM (OD_{600}) | SOT/yeast extract (OD_{600}) |
|-----------------|---------------|------------------------------|
| pSG464          | 0.051 (n = 3) | 0.431 (n = 3)                |
| pSG464-PAS8     | 1.352 (n = 2) | 1.311 (n = 2)                |
| pSG464-PAS8-PTS1R| 0.050 (n = 12)| 1.142 (n = 12)              |

The P. pastoris mutant pas8-1 (McCollum et al., 1993) was transformed by electroporation with the vector pSG464 (Gould et al., 1992) containing no insert, the complete PAS8 coding sequence, or a PAS8-PTS1R fusion construct. Arg-positive colonies were picked and grown overnight in YPD after which the cells were harvested, washed, and used as inoculum for SM or SOT/ye and allowed to grow for 48–72 h. Subsequently aliquots of the yeast cultures were harvested, washed, and resuspended in an equal volume of H_2O. Growth was assessed by determining the OD_{600}. Depicted are average values of a number of independent cultures. Note that the SOT medium was supplemented with 0.1% yeast extract explaining the growth observed in the pSG464 transformants. It was verified that a construct consisting of the NH_2-terminal half of PAS8p did not complement the pas8-1 mutant for growth on oleate or methanol.

PTS1R is Largely Cytosolic but Partly Localized to the Peroxisomal Compartment

A rat liver homogenate was fractionated by differential centrifugation into a soluble fraction, corresponding to the cytosol, and an organellar or light mitochondrial (A) fraction. The presence of PTS1R, catalase, and 3-ketocacyl-coenzyme A (CoA) thiolase in both of these fractions was examined by immunoblotting. PTS1R was predominantly found in the cytosol; only a minor amount could be detected in the organellar pellet (Fig. 6). The peroxisomal matrix enzymes catalase and thiolase, both known to easily leak from peroxisomes during fractionation, were found to be distributed equally over both fractions (Fig. 6), corroborating the existence of a genuine cytosolic PTS1R pool.

More, an immunoblot incubated with anticalatalase demonstrated that catalase was present in normal quantities in the Zellweger liver samples (data not shown).

PTS1R was present, and could be detected as a major band (Fig. 5 B, cf. lane 1 with lanes 2–5) by our antiserum, in whole cell lysates of various cultured cell lines derived from humans, monkey, and Chinese hamster. In most patient fibroblast cell lines PTS1R was present in normal or nearly normal quantities (Fig. 5 B, compare lane 5 with lanes 6, 7, and 9). However, in one Zellweger patient cell line, FAIR-T (Fig. 5 B, lane 8), belonging to complementation group two, the protein was absent. Note that the cells derived from an nALD patient ALA-T (Fig. 5 B, lane 7) belonging to the same complementation group contained a PTS1R protein of the normal size.

The P. pastoris mutant pas8-1 (McCollum et al., 1993) was transformed by electroporation with the vector pSG464 (Gould et al., 1992) containing no insert, the complete PAS8 coding sequence, or a PAS8-PTS1R fusion construct. Arg-positive colonies were picked and grown overnight in YPD after which the cells were harvested, washed, and used as inoculum for SM or SOT/ye and allowed to grow for 48–72 h. Subsequently aliquots of the yeast cultures were harvested, washed, and resuspended in an equal volume of H_2O. Growth was assessed by determining the OD_{600}. Depicted are average values of a number of independent cultures. Note that the SOT medium was supplemented with 0.1% yeast extract explaining the growth observed in the pSG464 transformants. It was verified that a construct consisting of the NH_2-terminal half of PAS8p did not complement the pas8-1 mutant for growth on oleate or methanol.

The Journal of Cell Biology, Volume 130, 1995 56
Table II. Comparison of Human PTS1R Sequences with Human Chromosomes in Human × Chinese Hamster Somatic Cell Hybrids

| Human chromosome | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X |
|------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---|
| Discordant hybrids |   |   |   |   |   |   |   |   |   | 2  | 1  | 3  | 2  | 1  | 4  | 1  | 4  | 1  | 2  | 0  | 5  | 6  | 3  | 2  | 2  | 3  | 4  | 1  | 5  | 4  | 2  |
| Condonant hybrids   |   |   |   |   |   |   |   |   |   | 4  | 3  | 6  | 5  | 3  | 6  | 1  | 4  | 2  | 1  | 5  | 8  | 4  | 6  | 5  | 0  | 5  | 7  | 6  | 6  | 6  | 1  |
| Informative hybrids | 15| 15| 14| 14| 16| 15| 14| 15| 14| 16| 14| 15| 16| 15| 16| 14| 16| 14| 16| 15| 15| 15| 15| 16| 16| 16| 13| 38| 33| 19| 47| 40| 43|
| Percent discordance | 33| 33| 29| 21| 38| 33| 57| 53| 36| 50| 29| 0  | 60| 50| 38| 29| 63| 38| 33| 19| 47| 40| 43|

The presence (+) or absence (−) of the PTS1R-specific PCR product is compared to the presence (+) or absence (−) of each chromosome in 16 independent hybrid cell lines. Data for chromosomes with rearrangements or present at low copy number (<0.1) were excluded. Percent discordance is calculated as the sum of discordant over total informative hybrids for each chromosome.

To characterize further the association of PTS1R with peroxisomes, we subfractionated highly purified peroxisomes by the one-step carbonate extraction procedure described by Fujiki et al. (1982). 3-Ketoacyl-CoA thiolase was confined to the soluble fraction, as expected for a soluble peroxisomal matrix protein (Fig. 7 C). Most of the PMP70, an integral membrane protein (Kamijo et al., 1990), was associated with the membranous fraction. The PTS1R protein, under these experimental conditions, appeared to be partially resistant to carbonate extraction, indicative of a very tight association with the peroxisomal membrane. With the PAS8 protein in P. pastoris, a large portion of the protein proved to be carbonate insoluble (Terlecky et al., 1995).

Additional evidence that PTS1R, in part, has a peroxisomal localization in mammalian cells was derived from double-labeling immunofluorescence experiments using CHO

Figure 4. Chromosomal mapping of the PTS1R gene. G-banding ideogram of human chromosome 12 with localization of human PTS1R gene by somatic cell hybrid analysis (SCH) and fluorescence in situ hybridization (FISH). Vertical bars represent the portions of chromosome 12 retained in two somatic-cell hybrid lines that were positive (+) or negative (−) for the human-specific PTS1 receptor PCR product.

Figure 5. Presence of PTS1R in liver homogenates and cultured cell lines from different organisms. (A) Liver extracts, corresponding to 100 μg of total protein, from a rat (lane 1), rat treated with the peroxisome proliferator clofibrate (lane 2), human (lanes 3 and 4), two unrelated Zellweger patients (lanes 5 and 6) were subjected to SDS-PAGE and transferred to nitrocellulose. The blot was incubated with anti-PTS1R serum. Molecular mass markers (kD) are indicated on the left. (B) Total cell extracts, corresponding to 100 μg of total protein, from rat liver (lane 1), human hepatoma cells (HepG2) (lane 2), African green monkey kidney cells (CV1) (lane 3), CHO cells (lane 4), control human skin fibroblasts BAS-T (lane 5), skin fibroblasts from patients suffering from peroxisomal disorders: GM3605-T (HPA, complementation group one, (lane 6), ALA-T (nALD, complementation group two, (lane 7), FAIR-T (ZS, complementation group two, (lane 8), and GM4340-T (ZS, complementation group four, (lane 9) were subjected to SDS-PAGE and blotted onto a nitrocellulose filter. The blot was incubated with anti-PTS1R serum. Molecular mass markers (kD) are indicated on the left.
Subcellular localization of PTS1R. Distribution of PTS1R, catalase, and 3-ketoacyl-CoA thiolase in a soluble (cytosolic) (S) and organelar (P) fraction. The fractions were prepared from rat liver homogenates by differential centrifugation as described by Keller et al. (1986). Equal portions of both fractions (corresponding to 79 μg of the soluble and 23.5 μg of the organelar fraction) were subjected to SDS-PAGE and the proteins transferred to nitrocellulose. Specific antisera were used to visualize the distribution of the proteins. Only the relevant parts of the gel are shown.

Figure 7. PTS1R is in part associated with peroxisomes. (A) Distribution profiles of the total amount of protein and marker enzyme activities after isopycnic centrifugation of a rat liver P fraction on a linear Nycodenz gradient: protein (■); esterase (ER, □); glutamate dehydrogenase (mitochondria, ○); phosphoglucone isomerase (cytosol, □); catalase (peroxisomes, ©). The ordinate, relative concentration (C/Ci), is derived by dividing the actual concentration of the enzyme in a particular fraction by the concentration of the enzyme that would be observed if the enzyme were homogeneously distributed throughout the gradient. The abscissa is the normalized cumulative volume (the total volume was 31 ml); the area of each graph is thus 1. Density of gradient increases from left to right. (B) Detection of PTS1R in different rat liver subcellular fractions. Proteins present in the fractions, characterized in A, were separated by SDS-PAGE and transferred electrotherically to nitrocellulose filters. The blot was subsequently incubated with anti-PTS1R. Molecular mass markers (kD) are indicated on the left. (C) Distribution of PTS1R, PMP70 and 3-ketoacyl-CoA thiolase in carbonate-extracted membranes and carbonate-soluble fraction of isolated rat liver peroxisomes. Highly purified peroxisomes were treated with 0.1 M Na2CO3 (pH 11.5) for 90 min at 0°C. After centrifugation a membrane pellet and a soluble fraction were obtained. Equal parts of the membrane fraction (P) and soluble fraction (S) (proteins of the carbonate soluble fraction were concentrated by trichloroacetic acid precipitation) were subjected to SDS-PAGE, blotted to nitrocellulose filters and incubated with anti-PTS1R, anti-PMP70 and anti-thiolase. Single bands of the expected molecular weights were detected. Only the relevant portions of the blot are shown. (D) Colocalization of PTS1R with SKL-containing proteins in semipermeabilized CHO cells. CHO cells were permeabilized with 0.2 U/ml streptolysin O, a condition in which much of the cytosol is retained in the cells, essentially as described by Wendland and Subramani (1993a). The semipermeabilized cells were then further processed for immunofluorescence and incubated in a first step with guinea pig anti-SKL antibody and rabbit anti-PTS1R, and in a second step with anti-guinea pig Ig rhodamine (a) and anti-rabbit Ig FITC (b).
Anti-PTS1R Antibodies Inhibit PTS1 Import

The ability of anti-PTS1R antibodies to interfere with the import of PTS1-containing proteins was tested by making use of an in vitro import system developed by Wendland and Subramani (1993a). The system uses streptolysin-O-permeabilized CHO cells and is extensively characterized using the substrate human serum albumin conjugated with a peptide (CRYHLKPLQSKL) ending in the peroxisomal targeting sequence SKL (HSA-SKL). The uptake of HSA-SKL into peroxisomes can be visualized by immunofluorescence. We examined the effect of anti-PTS1R on the uptake of HSA-SKL.

In a double-labeling experiment the HSA-SKL, under the conditions used, accumulated in a subcellular compartment (Fig. 8 B) which is identified as the peroxisome by its costaining with antibodies to catalase (Fig. 8 A). The addition of anti-PTS1R antiserum (Fig. 8 C) or an anti-PTS1R IgG preparation (Fig. 8 E) to the in vitro import system completely abolished the uptake of HSA-SKL, whereas the addition of preimmune serum (Fig. 8 D) or an equivalent amount of an unrelated IgG preparation had no effect (Fig. 8 F). These results show that PTS1R is required for peroxisomal protein import via the PTS1 (SKL-dependent) pathway, consistent with its role as the PTS1 receptor.

PTS1R Protein Binds to the SKL Targeting Signal

Two peptides, SKL (CRYHLKPLQSKL) and ΔSKL (CRY-HLKPLQ), were covalently coupled via their NH2-terminal cysteines to agarose beads. Both the ΔSKL and control (no peptide coupled) beads bound 25%–30% of the in vitro translated PTS1R (Fig. 9 A). When SKL beads were used, the amount of PTS1R bound to the beads increased to 85%, showing that it binds specifically to the SKL targeting signal. The addition of an excess of free SKL-peptide could compete the binding of PTS1R to SKL beads whereas the free ΔSKL or LKS (CRYHLKPLQLKS) peptides competed to a much lesser extent at similar concentrations (Fig. 9 B).

In the binding experiments we used a truncated PTS1R, missing 221 amino acids from its NH2-terminal end but encompassing its TPR domain, as we know from similar studies performed with the PAS8 protein that this portion of the protein is involved in the binding of the PTS1 (Terlecky et al., 1995). However, comparable results were obtained when a full-length PTS1R protein was used (data not shown).

PTS1R Restores the SKL Protein-import Deficiency in Fibroblasts from Patients with Fatal Peroxisomal Disorders Belonging to Complementation Group Two

We wished to determine whether a PTS1R deficiency could cause a generalized impairment of peroxisomal functions in the human group A disorders. Complementation group two contained two cell lines; one derived from a
mined by subjecting equal amounts of the supernatant and beads to SDS-PAGE and quantitating the amounts of radioactivity in each fraction using a Phosphorimager. The ability of PTS1R to bind to the beads was assessed as described in the Materials and Methods section. The percent distribution of bound (B) vs. unbound (S) PTS1R was determined in the presence of 2 μg (black bars) and 10 μg (hatched bars) of the following peptides: CRYHLKPLQSKL (SKL), CRYHLKPLQ (ΔSKL) and CRYHLKQLQK5 (LKS). Depicted is the percentage of bound PTS1R, as quantified by a phosphorimager, whereby the amount found to bind to SKL beads in the absence of any competing peptide was arbitrarily set at 100%.

Figure 9. PTS1R binds specifically to SKL-peptide. (A) The SKL peptide (CRYHLKPLQSKL) and the ΔSKL peptide (CRYHLKPLQ) were covalently coupled to Affigel 102 via the NH2-terminal cysteines on the peptides. The ability of PTS1R to bind to the beads was assessed as described in the Materials and Methods section. The percent distribution of bound (B) vs. unbound (S) PTS1R was determined by subjecting equal amounts of the supernatant and beads to SDS-PAGE and quantitating the amounts of radioactivity in each fraction using a Phosphorimager. (B) The ability of PTS1R to bind an SKL-peptide (CRYHLKPLQSKL), immobilized on agarose beads, was determined in the presence of 2 μg (black bars) and 10 μg (hatched bars) of the following peptides: CRYHLKPLQSKL (SKL), CRYHLKPLQ (ΔSKL) and CRYHLKQLQK5 (LKS). Depicted is the percentage of bound PTS1R, as quantified by a phosphorimager, whereby the amount found to bind to SKL beads in the absence of any competing peptide was arbitrarily set at 100%.

A full-length PTS1R mammalian cDNA expression construct injected into the nuclei of ALA-T and FAIR-T restored the ability of these cells to import SKL-containing proteins into their peroxisomes within 6 h after injection. This is illustrated in Fig. 11, E and F (ALA-T) and Fig. 11, G and H (FAIR-T), where E and G identify the microinjected cells stained for the presence of guinea pig antibodies that were coinjected. Sometimes the coinjected antibodies remained in the nucleus (Fig. 11 G) or dispersed through the cytoplasm (Fig. 11 E). The punctate fluorescence signal seen in the same cells stained with rabbit anti-SKL antibody (Fig. 11 F, ALA-T; Fig. 11 H, FAIR-T) is suggestive of import of SKL-containing proteins into the peroxisomes. Similar results were obtained when the group two cells were transfected, using the calcium phosphate precipitation technique, with the PTS1R expression construct. The SKL proteins colocalized with a peroxisomal integral membrane protein PMP70 (Fig. 12, C and D). The punctate labeling in the injected or transfected cells stained for SKL proteins was observed (Fig. 12 A) only when the plasma membrane and peroxisomes were permeabilized with digitonin (25 μg/ml) and Triton X-100 (1%), but not when the plasma membrane alone was permeabilized with digitonin (compare Fig. 12, A and B). These experiments prove that in group two cells injected with the PTS1R cDNA, the SKL proteins relocalize from the cytoplasm and reside within the peroxisome. Control experiments in which a Zellweger cell line from a different complementation group was injected never showed restoration of the punctate immunofluorescence pattern for SKL proteins (Fig. 11, D, I, and J). When the mammalian expression vector pJ7IL alone was injected into ALA-T or FAIR-T cells, no complementation of the mutant phenotype was observed. Based on these results, it is clear that the primary import defect in complementation group two disorders can be corrected by PTS1R protein.

In the FAIR-T cell line both PTS1 and PTS2 import pathways seem impaired (Figs. 10 E and 11 C). Upon microinjection or transfection of the PTS1R expression construct, the import of PTS2-containing proteins, i.e., 3-ketoacyl-CoA thiolase, was also restored as judged by the appearance of the characteristic punctate fluorescent pattern (Fig. 13). It was noted, however, that only some but not all transfected cells showed restoration of PTS2 import.

Discussion

The PTS1R Protein Is the Homologue of the P. pastoris PTS1 Receptor

Several lines of evidence suggest that the PTS1R protein is the human homologue of the PTS1 receptor. The PTS1R DNA exhibits 33.9% identity and 55.1% similarity to the P. pastoris PAS8 protein (Fig. 2). The PTS1R protein binds specifically to an SKL peptide (Fig. 9), as does PAS8 (McCollum et al., 1993). A PAS8-PTS1R fusion protein
complements the P. pastoris pas8 mutant for growth on oleate (Table 1). Both PAS8, and a small fraction of PTS1R (Fig. 7, B–D) (McCollum et al., 1993) are localized to peroxisomes, and a fraction of these proteins is tightly associated with the peroxisomal membrane. Finally, both PTS1R and PAS8 complement the selective protein-import deficiencies associated with the PTS1-pathway, in human and P. pastoris mutant cells, respectively. It is interesting to note that the PTS1R antibody recognizes a protein of the same size in human, rat, hamster, and monkey cells, suggesting that the protein is conserved in these species.

**Expression and Subcellular Location of PTS1R**

PTS1R RNA was found in all human tissues examined. This is not unexpected because peroxisomes comprise an essential metabolic compartment in eukaryotic cells. The PTS1R protein appeared to be highly soluble and was mainly recovered in a rat liver cytosolic fraction (Fig. 6). Only a small portion was present in a λ-fraction, enriched in peroxisomes. Analysis of the fractions of a rat liver peroxisome-purification gradient by Western blot analysis shows that most of the PTS1R loaded on the gradient is associated with the peroxisomes (Fig. 7 B). In addition, the PTS1R protein was localized to peroxisomes in CHO cells by indirect immunofluorescence (Fig. 7 D).

At least part of the PTS1R associated with the rat liver peroxisomes is insoluble in alkaline sodium carbonate (Fig. 7 C), suggesting that this fraction is tightly associated with the peroxisomal membrane. It is intriguing to speculate that PTS1R functions similarly to other proteins involved in protein transport, such as *Escherichia coli* Sec A and the signal recognition particle, which are known to be comprised of distinct pools that are cytosolic and transiently membrane-associated (Kim et al., 1994; Economou and Wickner, 1994; Luirink and Dobberstein, 1994).
PTS1R might shuttle between the cytosol and the peroxisome in performing its function as the PTS1 receptor. It seems clear from the data in Fig. 9 that soluble PTS1R is capable of binding the PTS1 peptide. The peroxisome-bound PTS1R may therefore be relevant to the delivery of PTS1-containing proteins to the translocation machinery on the peroxisomal membrane.

**Chromosomal Location of PTS1R**

PTS1R, the human PTS1 receptor, is assigned to chromosome 12p by somatic-cell-hybrid-mapping and independently confirmed and sublocalized to band 12p13.3 by FISH. No other known human peroxisomal disorders have yet been mapped to chromosome 12p. Three other genes associated with peroxisomal disorders have been mapped. First, peroxisomal membrane protein PXMP1 (PMP70), has been mapped to chromosome band 1p22-p21 (Gartner et al., 1993). Mutations in this gene have been found in patients with a generalized peroxisomal dysfunction belonging to complementation group one. The mouse homologue, Pmp-1, is located to chromosome 3 (Gartner et al., 1993).
Figure 12. SKL-containing proteins are imported into the peroxisomal matrix when cultured skin fibroblasts from group two peroxisomal disorders are complemented with PTS1R. Skin fibroblasts from an nALD patient, ALA-T as a representative of complementation group two were transfected with a full-length PTS1R expression construct. After 48 h the cells were fixed, permeabilized with 25 μg/ml digitonin and 1% Triton X-100 (A) or with 25 μg/ml digitonin alone (B), and stained with anti-SKL antibody. Alternatively, the transfected cells were processed for regular immunofluorescence and co-stained with guinea pig anti-PMP70 (C) and rabbit anti-SKL (D). Species-specific secondary antibodies conjugated to different fluorescent markers were used to detect bound primary antibodies. Bar, 20 μm.

1993). Second, PXMP3 (peroxisome assembly factor 1, PMP35), peroxisomal membrane protein-3, was mapped to chromosome region 8q21.1 (Masuno et al., 1994). A single Zellweger patient, belonging to complementation group F (see Shimozawa et al., 1993), was found to have a defective gene. Third, the human peroxisomal thiolase gene is located on chromosome 3p23-p22 (Bout et al., 1989) and was found deficient in 3-ketoacyl-CoA thiolase deficiency (pseudo-ZS). In addition, a microdeletion or inversion has been found in ZS patients: del (7) (7q11.12q11.23) and inv (7) (p12q11.23) (Naritomi et al., 1988, 1989).

Based on human-mouse comparative mapping data, we predict that the mouse homologue of the PTS1 receptor gene is located on mouse chromosome 6 in the region between 48 and 62 cM (Moore and Elliot, 1993). Two mutant loci associated with a neurological phenotype are within the region opisthotonus (opt) and deaf wddler (dfw) (Lane, 1972). The information available on these mutant phenotypes is too limited to evaluate them as possible mouse models.

Involvement of PTS1R in Peroxisomal Protein Import

The ability of PTS1R to bind SKL peptide (Fig. 9) and the inhibition of PTS1-specific import by PTS1R antibody (Fig. 8) show that PTS1R is an important component of the PTS1 protein-import pathway. We are developing new in vitro systems in which we hope to address whether PTS1R antibodies also inhibit the PTS2 import pathway, but these experiments are still in progress.

Role of PTS1R in Human Peroxisomal Disorders

The group two ALA-T cell line displays the same selective PTS1-import pathway deficiency as the P. pastoris pas8 and S. cerevisiae pas10 mutants (McCullum et al., 1993; Van der Leij et al., 1993). These yeasts and the human ALA-T mutant cell line represent the only ones characterized to date in which import via the PTS1 pathway is selectively affected. All the other group A disorders characterized (Motley et al., 1994), as well as a variety of yeast and CHO cell mutants (reviewed in Subramani, 1993), are defective in both the PTS1 and PTS2 import pathways. It is not yet clear whether these mutants represent defects in peroxisomal protein-import per se, or in the more general process of organelle biogenesis. The fact that in the FAIR-T cell line both PTS1 and PTS2 pathways seem to be affected might be related to the absence of the PTS1R protein in this cell line (Fig. 5 B). This observation raises the possibility of a connection between PTS1 and PTS2 import in mammalian cells. Interesting in this respect is the recent identification of PAS7 as the putative PTS2 receptor in S. cerevisiae by Marzoich et al. (1994). The PAS7p contains an amino acid sequence motif, the WD-40 repeat. Several members of the WD-40 family are known to interact with TPR proteins (Goebl and Yanagida, 1991; Van der Voorn and Ploegh, 1992).
The Journal of Cell Biology, Volume 130, 1995 64

The complementation of the PTS1-import defect in the
ZS and nALD cells from group two by the PTS1R cDNA,
the PTS1 import defect in these cells, and the absence
of the PTS1R protein in the ZS patient from group two argue
quite strongly that the group two cells are mutated in the
PTS1R gene. Although the genes (PMP70 and PAF-1) af-
fected in two other complementation groups of ZS pa-
tients are known (Gartner et al., 1992; Shimozawa et al.,
1992), nothing is known about the functions of these pro-
teins. Consequently, the molecular basis of the protein
import deficiency is not understood. The correction of the
import defect in the group two cell lines in our study
makes these the only peroxisomal disorders in which the
molecular basis of the protein-import defect is clearly un-
derstood. This study also represents one of the few exam-
ple cases in which a yeast gene led to the cloning of a human
gene that corrects two fatal human disorders at the cellular
level. It is hoped that this advance will lead, over time, to
rational ways with which therapies can be sought for these
devastating diseases.

Many thanks are due to Skai Krisans and Janis Schackelford (San Diego State University, San Diego, CA) for the preparation and characterization of the peroxisome-purification gradient in Fig. 7, to John Heyman for the anti-PMP70 antisera, to James Feramisco (University of California, San Diego, CA) for the use of the Microinjection Core Facility, to Gilbert Keller (Genentech, San Francisco, CA) for the guinea pig anti-PMP70 an-
tiserum, to Stanley Terlecky for the preparation of the SKL and SKL beads, to Drs. Ann and Hugo Moser for patient cell lines, and to Jon Singer for the use of his fluorescence microscope. During the course of this work we became aware that similar studies were being performed by Drs. Steve Gould and David Valle (Johns Hopkins University, Baltimore, MD). We appreciate their willingness to exchange some of the general re-

This study was supported by Human Frontier Science Program Organi-
ization (HFSPO) and American Heart Association fellowships to E. A. C. Wiemer, an HFSPO fellowship to W. M. Nuttley, a National Institutes of Health (NIH) postdoctoral fellowship to B. L. Bertolaet, grants CA44464 (to M. J. Wheelock) and GM41116 (to K. R. Johnson), an NIH research grant GM00298 (to U. Francke), the Howard Hughes Medical Institute of which U. Francke is an investigator, and X. Li is an associate, and grants from the NIH (DK41737) and National Science Foundation to S. Subra-
manian.

Note Added in Proof: Screening of available YACs from the distal 12p13 region revealed that the PTS1R gene is present in YACs 822_d_7, 944_b_1, 946_b_4, and 953_f_1. Given the STS content information for these YACs, we conclude that PTS1R maps near genes for the complement component C1r and for CD4 (Krauter, K., personal communication).

Received for publication 24 January 1995 and in revised form 4 April 1995.

References

Bout, A., J. M. N. Hoovers, E. Bakker, M. M. A. Mannens, A. Geurts van Kessel, A. Westerveld, J. M. Tager, and R. Benne. 1989. Assignment of the gene coding for human peroxisomal 3-oxoacyl-CoA thiolase (ACAA) to chromo-
some region 3p22-p23. Cytogenet. Cell Genet. 52:147-150.
Economou, A., and W. Wickner. 1994. SecA promotes preprotein translocation
by undergoing ATP-driven cycles of membrane insertion and deinsertion.
Cell 78:835-843.
Francke, U., T. L. Yang-Fang, J. E. Brissenden, and A. Ulrich. 1986. Chromo-
somal mapping of genes involved in growth control. Cold Spring Harbor Symp.
Quant. Biol. 51:855-866.
Frohman, M. A. 1990. RACE: rapid amplification of cDNA ends. In PCR Protocols: A Guide to the Methods and Applications. M. A. Innis, D. H. Gel-
faud, J. S. Sninsky, and T. J. White, editors. Academic Press, Inc., San Diego,
CA, 28-38.
Fujiki, Y., S. Fowler, H. Shio, A. L. Hubbard, and P. B. Lazaron. 1982. Polyphosphate and phospholipid composition of the membrane of rat liver per-
oxomes: comparison with endoplasmic reticulum and mitochondrial mem-
branes. J. Cell Biol. 93:103-110.
Gartner, J. W., W. Kears, C. Rosenberg, P. Pearson, N. G. Copeland, D. J. Gil-
bert, N. A. Jenkins, and D. Valle. 1993. Localization of the 70-kDa peroxiso-
mal membrane protein to human lp21-p22 and mouse 3. Genomics. 15:412-
414.
Gartner, J., H. W. Moser, and D. Valle. 1992. Mutations in the 70K peroxisomal
membrane protein gene in Zellweger syndrome. Nat. Genet. 1:16-22.
Goebel, M., and M. Yanaigida. 1991. The TPR snap helix: a novel protein repeat
motif from mitosis to transcription. TIBS (Trends Biochem. Sci.). 16:173-177.
Gould, S. J., G.-A. Keller, N. Hesken, J. Wilkinson, and S. Subramani. 1989. A
conserved tripeptide sorts proteins to peroxisomes. J. Cell Biol. 108:1657-
1664.
Gould, S. J., S. Krisans, G.-A. Keller, and S. Subramani. 1990. Antibodies di-
ruged against the peroxisomal targeting signal of firefly luciferase recognize
multiple mammalian peroxisomal proteins. J. Cell Biol. 110:27-34.
Gould, S. J., D. McCollum, A. P. Spong, J. A. Heyman, and S. Subramani. 1992.
Development of the yeast Pichia pastoris as a model organism for a genetic
and molecular analysis of peroxisome assembly. Yeast. 8:613-628.
Guan, K. L., and J. E. Dixon. 1991. Eukaryotic proteins expressed in
organisms: comparison with endoplasmic reticulum and mitochondrial mem-

Figure 13. Complementation of FAIR-T cells with PTS1R also
restores import of PTS2-containing proteins. Skin fibroblasts
from a ZS patient FAIR-T, belonging to complementation group
two, were transfected with a full-length PTS1R expression con-
struct. After 48 h the cells were processed for immunofluores-
cence and stained with anti-thiolase. Bar, 20 μm.
Kunau, W. H., and A. Hartig. 1992. Peroxisome biogenesis in Saccharomyces cerevisiae. Annu. Rev. Microbiol. 46:239-270.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680-685.

Lane, P. L. 1972. Two new mutations in linkage group XVI of the house mouse: flaky tail and varitint-waddler-J. Hered. 63:135-140.

Lazarow, P. B., and H. W. Moser. 1989. Disorders in peroxisome biogenesis. In The Metabolic Bases of Inherited Disease. 6th ed. C. R. Scriver, A. L. Beaudet, W. B. Sly, and D. Valle, editors. McGraw Hill Inc., New York, 6th edition. 1479-1589.

Li, X., P. Nghiem, H. Schulman, and U. Francke. 1994. Localization of the CAMKG gene encoding gamma isoforms of multifunctional calcium/calmodulin-dependent protein kinase (CaMK) to human chromosome 10 band q22 and mouse chromosome 14. Cytogenet. Cell Genet. 66:113-116.

Lurink, J., and B. Dobberstein. 1994. Mammalian and Escherichia coli signal recognition particles. Mol. Microbiol. 11:9-13.

Mannhaert, G. P., and P. P. van Velthoven. 1993. Metabolic pathways in mammalian peroxisomes. Biochimie (Paris). 75:147-158.

Marzioch, M., R. Erdmann, M. Veenhuis, and W. H. Kunau. 1994. PAS7 encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes. EMBO (Eur. Mol. Biol. Organ.) J. 13:4908-4918.

Masuno, M., N. Shimozawa, Y. Suzuki, N. Kondo, T. Orii, T. Tsukamoto, T. Osumi, Y. Fujiki, K. Imaizumi, and Y. Kuroki. 1994. Assignment of the human peroxisome assembly factor-1 gene (PaPM3) responsible for Zellweger syndrome to chromosome 6q21.1 by fluorescence in situ hybridization. Genomics 20:141-174.

McCullum, D., E. Monosov, and S. Subramaniam. 1993. The pas8 mutant of Pichia pastoris exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells: the PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is a member of the TPR protein family. J. Cell Biol. 121:761-774.

Militzovich, A., J. Travis, R. Grosschedl, and U. Francke. 1991. Gene for lymphoid enhancer-binding factor 1 (LEF1) mapped to human chromosome 4 (q23-q25) and mouse chromosome 3 near Egl. Genomics. 11:1084-1098.

Moore, K. J., and R. W. Elliott. 1993. Mouse chromosome 6. Mamm. Genome. 4:888-8109.

Morgenstern, J. P., and H. Land. 1990. A series of mammalian expression vectors and characterization of their expression of a reporter gene in stably and transiently transfected cells. Nucleic Acids Res. 18:1068.

Motley, A., E. Hettema, B. Dietel, and H. F. Tabak. 1994. Differential protein import deficiencies in human peroxisome assembly disorders. J Cell Biol. 125:755-767.

Naritomi, K., N. Hyakuna, Y. Suzuki, T. Orii, and K. Hirayama. 1988. Zellweger syndrome and a microdeletion of the proximal long arm of chromosome 7. Hum. Genet. 80:201-202.

Naritomi, K., Y. Izuikuma, S. Ohshiro, K. Yoshida, N. Shimozawa, Y. Suzuki, T. Orii, and K. Hirayama. 1989. Gene assignment of Zellweger syndrome to 7q11.23: report of the second case associated with a pericentric inversion of chromosome 7. Hum. Genet. 84:79-80.

Osumi, N., T. Tsukamoto, S. Hata, Y. Fujiki, M. Hiijikata, S. Miyazawa, and T. Hashimoto. 1991. Amino-terminal premature cleavage of the precursor of peroxisomal 3-ketoacyl-CoA thiolase is a cleavable signal peptide for peroxisonal targeting. Biochem. Biophys. Res. Commun. 181:947-954.

Parker, B. A., and G. R. Stark. 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by vi- rus or viral DNA. J. Virol. 31:360-369.

Roscher, A. A., S. Hoefler, G. Hoefler, E. Paschke, F. Paltauf, A. Moser, and H. Moser. 1989. Genetic and phenotypic heterogeneity in disorders of peroxisome biogenesis: a complementation study involving cell lines from 19 patients. Pediatr. Res. 26:67-72.

Santos, M. J., T. Imanaka, H. Shio, and P. B. Lazarow. 1988a. Peroxisomal integral membrane proteins in control and Zellweger fibroblasts. J. Biol. Chem. 263:10502-10509.

Santos, M. J., T. Imanaka, H. Shio, G. M. Small, and P. B. Lazarow. 1988b. Peroxisomal membrane ghosts in Zellweger syndrome: aberrant organelle assembly. Science (Wash. DC). 239:1536-1538.

Shimozawa, N., T. Tsukamoto, Y. Suzuki, T. Orii, Y. Shirayoshi, T. Mori, and Y. Fujiki. 1992. A human gene responsible for Zellweger syndrome that affects peroxisome assembly. Science (Wash. DC). 255:1132-1134.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85.

Subramaniam, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. Annu. Rev. Cell Biol. 9:445-478.

Swinkels, B. W., S. J. Gould, A. G. Bodnar, R. A. Rachubinski, and S. Subramaniam. 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. EMBO (Eur. Mol. Biol. Organ.) J. 10:3255-3262.

Terlecky, S. R., W. M. Nuttley, D. McCollum, E. Sock, and S. Subramaniam. 1995. The Pichia pastoris peroxisomal protein, PAS8p, is the receptor for the carboxy-terminal, tripeptide peroxisomal targeting signal. EMBO (Eur. Mol. Biol. Organ.) J. In press.

Towbin, H., T. Stachelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

Van den Bosch, H., R. B. H. Schutgens, R. J. A. Wanders, and H. F. Tager. 1992. Biochemistry of peroxisomes. Annu. Rev. Biochem. 61:157-197.

Van der Leij, I., M. M. Fransse, Y. Elgersma, B. Distel, and H. F. Tabak. 1993. Pasl0 is a tetratricopeptide-repeat protein that is essential for the import of most matrix proteins into the peroxisomes of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. 90:11782-11786.

Van der Veen, L., and H. L. Ploegh. 1992. The WD-40 repeat. FEBS (Fed. Eur. Biochem Soc.) Lett. 307:131-134.

Walton, F. A., S. J. Gould, J. R. Feramisco, and S. Subramaniam. 1992. Transport of microinjected proteins into peroxisomes of mammalian cells: inability of Zellweger cell lines to import proteins with the SKL tripeptide peroxisomal targeting signal. Mol. Cell. Biol. 12:531-541.

Wanders, R. J. A., H. S. A. Heyman, R. B. H. Schutgens, P. G. Barth, H. van den Bosch and J. M. Tager. 1988. Peroxisomal disorders in neurology. J. Neurol. Sci. 88:1-39.

Wendland, M., and S. Subramaniam. 1993a. Cytochrome c-dependent peroxisomal protein import in a permeabilized cell system. J. Cell Biol. 120:675-685.

Wendland, M., and S. Subramaniam. 1993b. Presence of cytoplasmic factors functional in peroxisomal protein import implicates organelle-associated defects in several human peroxisomal disorders. J. Clin. Invest. 92:2462-2468.

Wiemer, E. A., S. Brul, W. W. Just, R. Van Driel, E. M. Brouwer-Kelder, M. van den Berg, P. J. Weijers, R. B. H. Schutgens, H. van den Bosch, A. W. Schram et al. 1989. Presence of peroxisomal membrane proteins in liver and fibroblasts from patients with the Zellweger syndrome and related disorders: evidence for the existence of peroxisomal ghosts. Eur. J. Cell Biol. 50:407-417.