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

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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 *Pichia pastoris PAS8* gene, the PTS1 receptor (McCollum, D., E. Monosov, and S. Subramani. 1993. *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 PAS8-PTS1R fusion protein complements the *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).[1] 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; Mannaerts 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), hyperpipecolic acidemia (HPA), the neonatal form of adrenoleukodystrophy (nALD) and infantile Refsum's disease. Patients suffering from ZS are clinically characterized by severe craniofacial dysmorphisms, 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).

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1. *Abbreviations used in this paper*: CoA, coenzyme A; FISH, fluorescence in situ hybridization; GST, glutathione-*S*-transferase; HPA, hyperpipecolic acidemia; HSA, human serum albumin; nALD, neonatal adrenoleukodystrophy; PMP, PXMP, peroxisomal membrane protein; PTS, peroxisomal targeting signal; SKL, serine-lysine-leucine; TPR, tetratricopeptide repeat; ZS, cerebro-hepato-renal (Zellweger) syndrome.

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., 1988*a*,*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, 1993*b*; 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* (McCollum 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; Marzoich 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 (McCollum 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% bactopeptone, 2% glucose). Defined synthetic medium (S) consisted of yeast nitrogen base at 6.7 g/l supplemented with carbon sources to a final concentration of either 2% glucose (SD), 0.5% methanol (SM), or 0.2% oleic acid/0.02% Tween-40 (SOT). Arginine was added as needed to a final concentration of 50 μg/ml. Solid media were prepared by adding 2% bactoagar before autoclaving. The *P. pastoris pas8-1* mutant has been characterized by McCollum et al. (1993).

Cultured human skin fibroblasts were obtained from patients with ZS (cell lines FAIR-T and GM4340-T, complementation groups two and four, respectively); nALD (cell line ALA-T, complementation group two); HPA (cell line GM3605-T, complementation group one). The cell lines PUCK and BAS-T were used as controls, and not derived from patients with peroxisomal disorders. All cell lines, except the control cell line PUCK, were immortalized by transfection with pRSV-SV40T and selection for foci (see Wendland and Subramani, 1993*b*). The cell lines GM3605 and GM4340 were obtained from the Human Mutant Cell Culture Repository (Camden, NJ); the other cell lines were obtained from Drs. Ann and Hugo Moser (Kennedy Krieger Institute, Baltimore, MD). The complementation group designation is as published by Roscher et al. (1989). For a standardization of the complementation grouping of peroxisome-deficient disorders see Shimozawa et al. (1993). CHO, CV1, HepG2 cells are from American Type Culture Collection (Rockville, MD).

Autopsy Material

Liver samples from two patients with ZS (unknown complementation group) and from two controls were kindly supplied by Dr. Hugo Moser. Liver homogenates were prepared as described by Krisans et al. (1994).

Antibodies

Anti–PTS1R antibodies were raised as follows: a HindII-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-Cl, pH 8.0, 150 mM NaCl; 2.5 mM $CaCl_2$ 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-Coenzyme A 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 antisera directed against HSA were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Species-specific anti-Ig 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 transcriptase-PCR (RT-PCR), essentially according to Frohman (1990). Poly(A^+) human liver mRNA (Clontech Laboratories, Inc.) was used as template in the reverse transcriptase reaction in combination with the gene specific primer 5′GTGAACTGGTCAACCCAGGC (nucleotides 674–655). The 5′end of PTS1R was PCR amplified using a second, nested, gene-specific primer 5′CAGCGATCGGTGGCTGTTCC (nucleotides 551–532) and the anchor primer 5′GACTCGAGTCGACATCGAT_{17}. The PCR fragment was cloned into the pCR™II cloning vector (Invitrogen, San Diego, CA). Both strands were sequenced. A full-length PTS1R construct was prepared in pBSKSII (Stratagene, Inc. La Jolla, CA) creating pBS-PTS1R.

PTS1R Plasmid Constructs

An NcoI-BglII (nucleotides −2–2050) fragment was isolated from pBS-PTS1R and the NcoI site overlapping the translation start codon was filled in using Klenow polymerase thereby restoring the ATG. The fragment, which encompasses the complete PTS1R coding sequence, was cloned into the SmaI and BglII polylinker sites of the mammalian expression plasmid pJ7Ω under the control of the cytomegalovirus promoter (Morgenstern and Land, 1990). The resulting pJ7Ω-PTS1R was used in the nuclear microinjection and transfection experiments.

A HindII-BglII (nucleotides 663–2050) fragment was cloned in-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 NH_2-terminal 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 NH_2–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 HindII-XhoI (nucleotides 663–1534) fragment from PTS1R was uniformly labeled with [α^{32}P]dCTP 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) lowfat 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).

The one-step carbonate procedure as described by Fujiki et al. (1982) was used to prepare a membranous fraction containing only integral membrane proteins. In brief, highly purified rat liver peroxisomes were diluted at least 16-fold with 0.1 M Na_2CO_3, pH 11.5, to a final protein concentration of 1.15 mg/ml, incubated on ice for 90 min, and centrifuged for 90 min at 100,000 *g*, yielding a membrane pellet and a soluble fraction.

Protein concentration was determined according to Smith et al. (1985).

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-SKL as a substrate (Wendland and Subramani, 1993*a*). Import was assessed by immunofluorescence microscopy with polyclonal antisera. HSA was detected using a rabbit anti-HSA serum at a 1:250 dilution. For double labeling, guinea pig anti-HSA and rabbit anti–catalase antisera were used at a 1:500 and 1:100 dilution, respectively. Bound primary antibodies were detected by FITC- and rhodamine-conjugated secondary antibodies. 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 anti-PTS1R or nonspecific sera by protein A chromatography (Harlow and Lane, 1988) and used in import reactions at 1.5 μg/μl.

Immunofluorescence Microscopy

Immunofluorescence microscopy was essentially performed as described by Keller et al. (1987). Cultured skin fibroblasts were permeabilized selectively by digitonin as described by Swinkels et al. (1991).

PTS1R Binding Studies

The SKL peptide (CRYHLKPLQSKL) and the ΔSKL peptide (CRYHLKPLQ) were coupled to Affigel 102 (Bio-Rad Laboratories) via the NH_2-terminal cysteines on the peptides, according to the manufacturer's specifications. 15 μl of in vitro translated PTS1R protein were mixed with 35 μl of binding buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, and 2 mM DTT) which was then added to a 50-μl vol of bead conjugate and incubated for 1 h at room temperature with frequent mixing. In peptide competition experiments 2 μg and 10 μg of each of the following peptides: SKL peptide, ΔSKL peptide, and LKS peptide (CRYHLKPLQLKS) were added to the incubation. The supernatant, containing unbound PTS1R protein, was removed and saved. The beads were washed five times with 1 ml of binding buffer and then suspended in 50 μl of binding buffer. SDS-PAGE sample buffer was added to the fractions containing bound (beads) and unbound PTS1R, heated to 100°C for 5 min, and equivalent fractions of the supernatant and beads were subjected to SDS-PAGE. The distribution of PTS1R was examined by fluorography and the signals quantified by exposing the gels to a Phosphorimager screen (Molecular Dynamics, Inc., Sunnyvale, CA). All solutions used in the binding assay contained the following protease inhibitors: 5 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 0.2 mM PMSF.

Somatic Cell Hybrid Panel and PCR Amplifications

We used a mapping panel of 16 human × Chinese hamster hybrid cell lines containing overlapping sets of human chromosomes for primary assignment of the PTS1 receptor gene to a human chromosome. Two hybrid cell lines with partial deletions of chromosome 12 were used for regional mapping (summarized in Francke et al., 1986). Genomic DNAs from the panel were amplified using PTS1 receptor-specific primers designed from the 3′untranslated region of the gene. The forward primer was 5′-CGCCTCTAATGTCTGTCTGGATGG (nucleotides 2247–2271) and the reverse primer 5′-TTCCAATGCTCTCCCCTAGAAAC (nucleotides 2787–2765), the amplification product was 541 bp in size. The PCR conditions were 95°C, 5 min; then 35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min; followed by 72°C, 7 min.

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 cDNA probe was hybridized at a concentration of 400 ng/50 μl per slide to pretreated and denatured metaphase chromosomes from a human peripheral blood lymphocyte culture in the presence of sheared unlabeled 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 Na_2PO_4, 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 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

using the calcium phosphate precipitate technique described by Parker and Stark (1979).

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 RT-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 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 tetratricopeptide repeat (TPR) protein family to which proteins with diverse functions (chromosome segregation, cell cycle control, mitochondrial import, and transcription) belong (Goebl 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 NH_2-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 PAS8–PTS1R fusion protein (the NH_2-terminal part of PAS8p linked to the TPR domain of PTS1R) to complement the *P. pastoris pas8-1* 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 *pas8-1* mutant to grow on media containing oleate as sole C-source, however, it did not restore the ability to metabolize methanol.

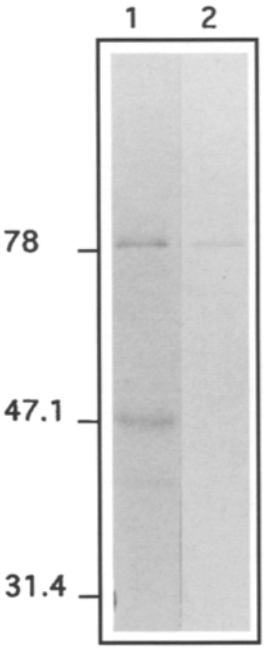

Figure 1. In vitro translated PTS1R comigrates with rat liver PTS1R. Radiolabeled full-length PTS1R, prepared by in vitro transcription-translation reactions, was mixed with a rat liver extract, corresponding to 100 μg total protein, and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose and the [^{35}S]methionine-labeled PTS1R detected by autoradiography (lane *1*). The blot was then incubated with anti-PTS1R serum to visualize the PTS1R present in the rat liver homogenate (lane *2*). Molecular mass markers (kD) are indicated on the left.

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.

```
          1
HsPTS1R   MAMRELVEAE CGGANPLMKL AGHFTQDKAL RQEGLRPGPW PPGAPASEAA
 PpPAS8   MSLIGGGSDC AAGSNPLAQF TKHTQHDTSL .QQSMRNGEF QQGNQRMMRN
ScPAS10   ...MDVGS.C SVGNNPLAQL HKHTQQNKSL .QFNQKNNGR LNESPLQGTN
                     * ***      *      *  *

          51
HsPTS1R   SKPLGVASED ELVAEFLQDQ NAPLVSRAP. .QTFKMDDLL AEMQQIEQS.
 PpPAS8   ESTMSPMERQ Q.MDQFMQQQ NNPAFNFQPM QHELNV..MQ QNMNAPQQVA
ScPAS10   KPGISEAFIS N.VNAISQE. .....NMANM QRFINGEPLI DDKRRMEIGP
                          *

          98
HsPTS1R   .......... .NFRQAPQRA PGVADLALSE NWAQEFLAAG DAVDVTQDYN
 PpPAS8   NNSWNQEFRM KDPMVANAPS AQVQTPVQST NWAQDFQQAG PEVQHHAQQH
ScPAS10   SSGRLPPFSN VHSLQTSANP TQIKGVNDIS HWSQEFQGSN SIQNRNADTG
                                           * * *

          137
HsPTS1R   ETDWS..... .......... ....QEFISE VTDPLSVSPA RWAEEYLEQS
 PpPAS8   QHPILSVPGV RAG....... .......IYG GGRLMGGSMM NRAAQMQQQN
ScPAS10   NSEKAWQRGS TTASSRFQYP NTMMNNYAYA SMNSLSGSRL QSPAFMNQQQ
                                                    *          *

          168
HsPTS1R   EEKLWLGEPE GTATDRWYDE YHPEEDLQHT ASDFVAKVDD PKLANSEGTS
 PpPAS8   PAQAQTS..E QSQTQW.... ...EDQFKDI E......... ..........
ScPAS10   SGRSKEGVNE QEQQPW.... ...TDQFEKL EKEVSENLDI NDEIEKEENV
               *

          218
HsPTS1R   DAWVDQFTRP VNTSALDMEF ERAKSAIESD VDFWDKLQAE LEEMAKRDAE
 PpPAS8   SM.LNSKTQE PKTKQQE... ...QNTFE.. .QVWDDIQVS YADV...ELT
ScPAS10   SE.VEQNKPE TVEKEEGVYG DQYQSDFQ.. .EVWDSIHKD AEEVLPSELV
                                             **

          268
HsPTS1R   AHPWLSDYDD ......LTSA TYDKGYQFEE ENPLRDHPQP FEEGLRRLQE
 PpPAS8   NRPVSGSMGE RFCPIRRRRL NYG.EYKYEE KNQFRNDPDA YEIGMRLMES
ScPAS10   NDDL..NLGE DYLKYLGGRV NGNIEYAFQS NNEYFNNPNA YKIGCLLMEN
                                      *      *     *      *

          312
HsPTS1R   G.DLPNAVLL FEAAVQQDPK HMEAWQYLGT TQAENEQELL AISALRRCLE
 PpPAS8   GAKLSEAGLA FEAAVQQDPK HVDAWLKLGE VQTQNEKESD GIAALEKCLE
ScPAS10   GAKLSEAALA FEAAVKEKPD HVDAWLRLGL VQTQNEKELN GISALEECLK
          *  *  * *  *****   *  *  **  **   *  ** *    * **  **

          361
HsPTS1R   LKPDNQTALM ALAVSFTNES LQRQACETLR DWLRYTPAYA HLVTPAEEGA
 PpPAS8   LDPTNLAALM TLAISYINDG YDNAAYATLE RWI..ETKYP DIASRARSSN
ScPAS10   LDPKNLEAMK TLAISYINEG YDMSAFTMLD KWA..ETKYP EIWSRIKQQD
          * * *  *    ** *  *       *   *   *      *

          411
HsPTS1R   GGAGLGPSKR ILGSLLSDSL FLEVKELFLA AVRL..DPTS IDPDVQCGLG
 PpPAS8   PDLD...GGD RIE......Q NKRVTELFMK AAQLSPDVAS MDADVQTGLG
ScPAS10   DKFQKEKGFT HID......M NAHITKQFL. ..QLANNLST IDPEIQLCLG
                                      *     *        *   *  **

          459
HsPTS1R   VLFNLSGEYD KAVDCFTAAL SVRPNDYLLW NKLGATLANG NQSEEAVAAY
 PpPAS8   VLFYSMEEFD KTIDCFKAAI EVEPDKALNW NRLGAALANY NKPEEAVEAY
ScPAS10   LLFYTKDDFD KTIDCFESAL RVNPNDELMW NRLGASLANS NRSEEAIQAY
           **      * *  ***  **  * *   * * * *** ***  *  ***  **

          509
HsPTS1R   RRALELQPGY IRSRYNLGIS CINLGAHREA VEHFLEALNM Q.....RKSR
 PpPAS8   SRALQLNPNF VRARYNLGVS FINMGRYKEA VEHLLTGISL HEVEGVD.AS
ScPAS10   HRALQLKPSF VRARYNLAVS SMNIGCFKEA AGYLLSVLSM HEVNTNNKKG
           *** * *    * **** *   * *   **      *

          554
HsPTS1R   GPRGEGGAMS ENIWSTLRLA LSMLGQSDAY GAA.DARDLS TLLTMFGLPQ
 PpPAS8   EMSSNQGLQN NALVETLKRA FLGMNRRDLV DKVYPGMGLA QFRKMFDF
ScPAS10   DVGSLLNTYN DTVIETLKRV FIAMNRDDLL QEVKPGMDLK RFKGEFSF
                       **           *           *       *
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Figure 2. Sequence alignment between the human PTS1R (*HsPTS1R*), *P. pastoris* PAS8 (*PpPAS8*), and *S. cerevisiae* PAS10 (*ScPAS10*) using the PILEUP program. Identical residues are indicated by an asterisk below the sequence. The TPR domain of PTS1R is underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z48054.

These results indicate that the PTS1R gene is located on the short arm of chromosome 12 (Fig. 4).

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 chromatids 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_20 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.

rat liver and human liver homogenates, revealed one major protein of 80 kD that was recognized by an anti-PTS1R serum (Fig. 5 *A*, lanes *1–4*). The apparent discrepancy between the predicted molecular mass of PTS1R (67 kD) and the immunologically identified protein can be explained by assuming that PTS1R migrates aberrantly on SDS–polyacrylamide gels. Indeed in subsequent experiments we showed that in vitro translated PTS1R comigrates with the 80-kD protein that is recognized by the antiserum (Fig. 1), identifying this protein as PTS1R. The 80-kD protein did not seem to be induced significantly when rats were treated with the hypolipidemic drug clofibrate, which is known to cause induction of the peroxisomal β-oxidation system and peroxisomal proliferation (Fig. 5 *A*, compare lanes *1* and *2*). However, the protein was absent or markedly reduced in the two liver homogenates from ZS patients (Fig. 5 *A*, lanes *5* and *6*) indicating that the 80-kD protein is indeed related to the peroxisomal compartment. Nonspecific proteolytic breakdown of the Zellweger autopsy liver material as an explanation for the absence of PTS1R is unlikely as the Ponceau S staining profile after blotting revealed no general protein degradation. Furthermore, an immunoblot incubated with anticatalase 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.

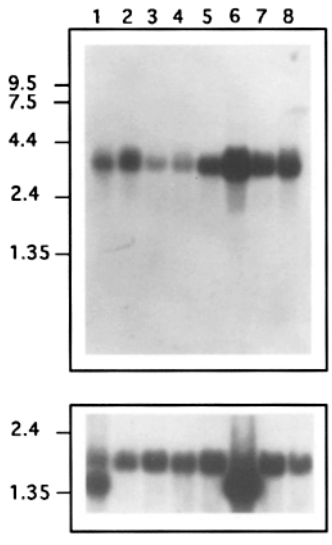

Figure 3. Expression of the PTS1R gene in various human tissues. A multiple human tissue Northern blot contains ~2 μg of poly(A^+) RNA per lane from heart (lane *1*); brain (lane *2*); placenta (lane *3*); lung (lane *4*); liver (lane *5*); skeletal muscle (lane *6*); kidney (lane *7*); and pancreas (lane *8*). The top panel shows the blot incubated with a PTS1R-specific probe, the bottom panel shows the blot incubated with a control human β-actin cDNA probe. A 2-kb β-actin mRNA was observed in all lanes and in heart and skeletal muscle an additional smaller mRNA of 1.6–1.8 kb representing a β-actin isoform that is expressed in these tissues. Markers in kilobases are depicted on the left.

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 (λ) fraction. The presence of PTS1R, catalase, and 3-ketoacyl-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.

The organellar fraction shown in Fig. 6 was further fractionated by isopycnic centrifugation on a continuous Nycodenz gradient. The gradient was characterized by measuring protein content and marker enzyme activities for different subcellular organelles (Fig. 7 *A*). Peroxisomes peak, as judged by the distribution of catalase, in the dense part of the gradient (fractions 17–22), well separated from microsomal and mitochondrial fractions (Fig. 7 *A*). A small part of the catalase activity was observed at the top of the gradient, comigrating with the cytoplasmic marker enzyme phosphoglucose isomerase. This represents catalase released from the peroxisomes during fractionation. To reproduce the distribution of different subcellular organelles in the gradient, equal portions of each fraction were subjected to SDS-PAGE and blotted onto nitrocellulose. The blot was incubated with the anti-PTS1R serum. Most of the PTS1R loaded on the gradient was present in the peroxisomal peak fractions (Fig. 7 *B*). The lower mol wt bands observed are most likely degradation products of PTS1R. A considerable trailing into the lighter part of the gradient was observed, a phenomenon which is not uncommon for peroxisomal membrane or membrane-associated proteins. The PMP70 distribution profile colocalized with the peroxisomes. Although the trailing was less pronounced than observed with PTS1R, some PMP70 was present in the mitochondrial peak fractions (data not shown). It might be that PTS1R, which has no clearly identifiable membrane anchoring–domain, is released from the peroxisomes as they move through the gradient to their buoyant density.

Table II. Comparison of Human PTS1R Sequences with Human Chromosomes in Human × Chinese Hamster Somatic Cell Hybrids

PTS1R Signal/chromosome	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																							
+/−	3	4	1	1	5	1	7	4	4	7	2	0	4	2	3	2	8	3	1	2	2	2	1
−/+	2	1	3	2	1	4	1	4	1	1	2	0	5	6	3	2	2	3	4	1	5	4	2
Concordant hybrids																							
+/+	4	3	6	5	3	6	1	4	2	1	5	8	4	6	5	6	0	5	7	6	6	6	1
−/−	6	7	4	6	7	4	5	3	7	7	5	8	2	2	5	4	6	5	3	7	2	3	3
Informative hybrids	15	15	14	14	16	15	14	15	14	16	14	16	15	16	16	14	16	16	15	16	15	15	7
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 rearrangments 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

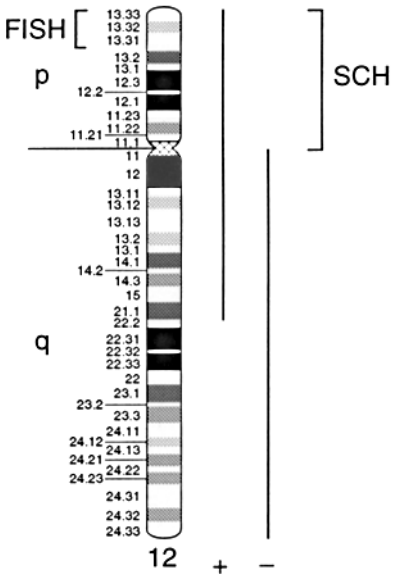

Figure 4. Chromosomal mapping of the PTS1R gene. G-banding ideogram of human chromosome 12 with localization of human PTS1R gene by somatic cell hybrid analysis (*SCH*) and fluorescence in situ hybridization (*FISH*). Vertical bars represent the portions of chromosome 12 retained in two somatic-cell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

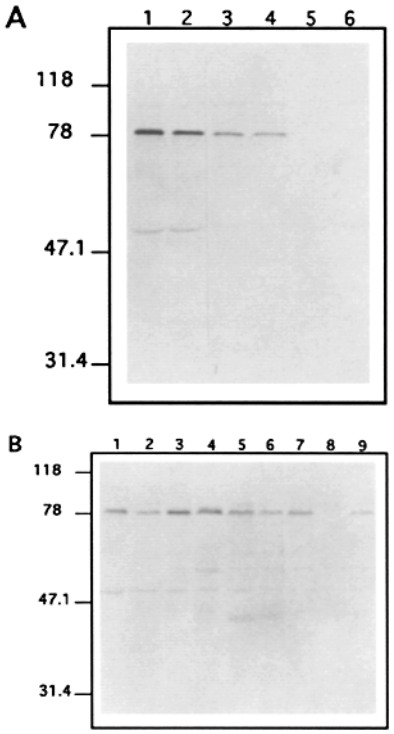

Figure 5. Presence of PTS1R in liver homogenates and cultured cell lines from different organisms. (*A*) Liver extracts, corresponding to 100 μg of total protein, from a rat (lane *1*), rat treated with the peroxisome proliferator clofibrate (lane *2*), human (lanes *3* and *4*), two unrelated Zellweger 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.

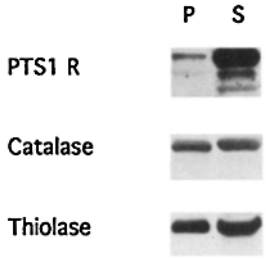

Figure 6. Subcellular localization of PTS1R. Distribution of PTS1R, catalase, and 3-ketoacyl-CoA thiolase in a soluble (cytosolic) (*S*) and organellar (λ-fraction) fraction (*P*). The fractions were prepared from rat liver homogenates by differential centrifugation as described by Keller et al. (1986). Equal portions of both fractions (corresponding to 79 μg of the soluble and 23.5 μg of the organellar 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.

cells. A punctate fluorescent pattern was observed in CHO cells incubated with an anti-SKL antiserum identifying the peroxisomal compartment (Fig. 7 *D*, *a*). A similar pattern, which is in fact completely superimposable with the anti-SKL signal, was found when the anti-PTS1R signal was observed (Fig. 7 *D*, *b*). This peroxisomal localization of PTS1R by immunofluorescence was observed only in streptolysin-*O*–permeabilized cells, and not in cells fixed and permeabilized without initial streptolysin-*O* treatment. Although the streptolysin-*O* permeabilization conditions do not allow all the cytosol to leak out (Wendland and Subramani, 1993*a*), it is possible that some of the cytosolic PTS1R is released, revealing the peroxisome-associated PTS1R more clearly.

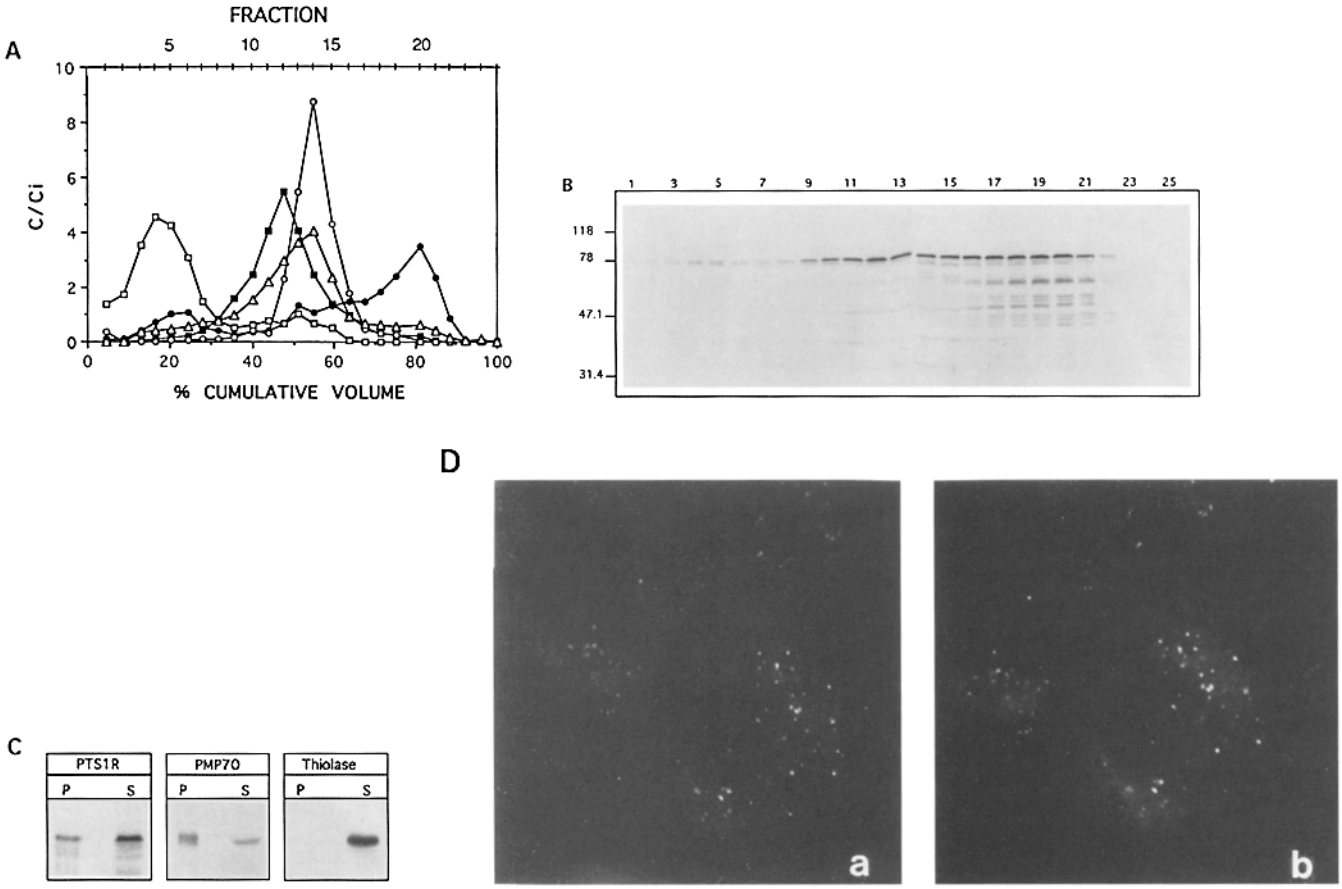

Figure 7. PTS1R is in part associated with peroxisomes. (*A*) Distribution profiles of the total amount of protein and marker enzyme activities after isopycnic centrifugation of a rat liver λ-fraction on a linear Nycodenz gradient: protein (△); esterase (ER, ■); glutamate dehydrogenase (mitochondria, ○); phosphoglucose 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 electrophoretically 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 Na_2CO_3 (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 (1993*a*). 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*).

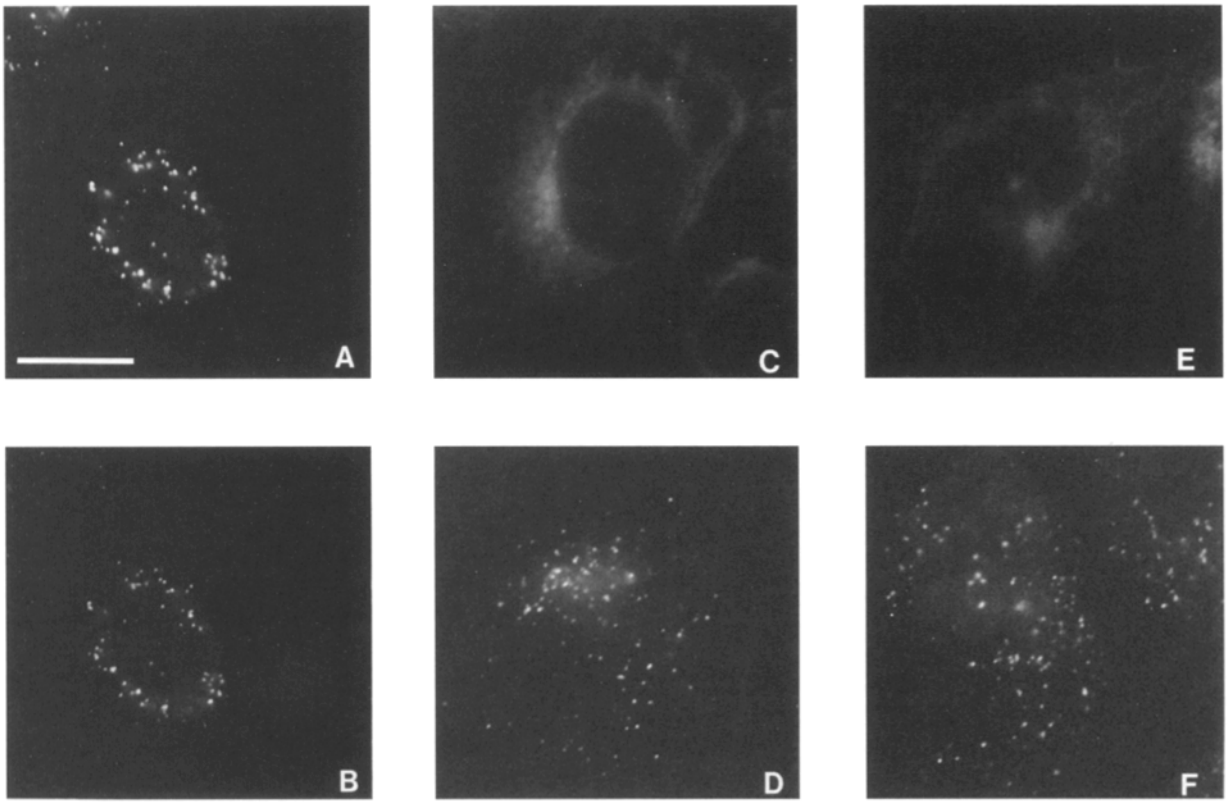

Figure 8. Anti-PTS1R antibody inhibits import of HSA-SKL into semipermeabilized CHO cells. An in vitro import assay (Wendland and Subramani, 1993*a*) was performed using streptolysin-*O* permeabilized CHO cells and 50 μg/ml HSA-SKL as a substrate. Import was assessed by immunofluorescence microscopy with polyclonal antisera. *A* and *B*: double labeling experiment in which permeabilized cells were stained with rabbit anti-catalase (*A*) and guinea pig anti-HSA (*B*). *C–F*: import was performed in the presence of 5 μl anti-PTS1R serum (*C*), 5 μl preimmune serum (*D*), 1.5 μg/μl of an anti-(PTS1R) IgG preparation (*E*), or 1.5 μg/μl of an unrelated IgG preparation (*F*). The uptake of HSA was determined with rabbit anti-HSA. Bound primary antibodies were detected by FITC- and rhodamine-conjugated secondary antibodies. Bar, 20 μm.

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 (1993*a*). 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 (CRYHLKPLQ), were covalently coupled via their NH_2-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 NH_2-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

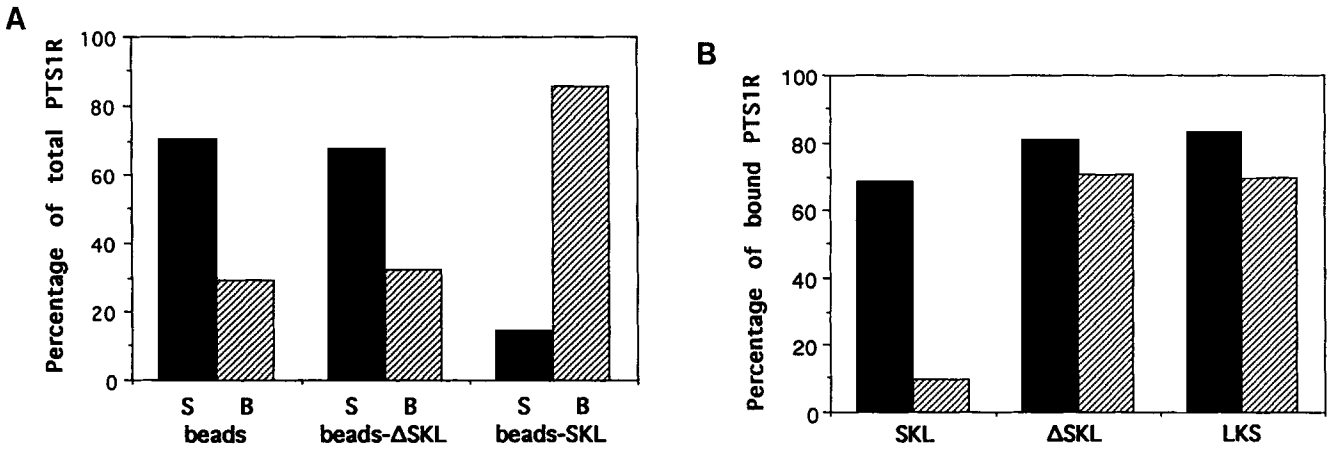

Figure 9. PTS1R binds specifically to SKL-peptide. (*A*) The SKL peptide (CRYHLKPLQSKL) and the ΔSKL peptide (CRYHLKPLQ) were covalently coupled to Affigel 102 via the NH_2-terminal cysteines on the peptides. The ability of PTS1R to bind to the beads was assessed as described in the Materials and Methods 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 CRYHLKPLQLKS (*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%.

patient suffering from nALD (ALA-T) and another derived from a ZS patient (FAIR-T). Both cell lines were informative with regard to PTS1R. The ALA-T cell line displayed a phenotype that was reminiscent of the phenotype of the *P. pastoris pas8* and *S. cerevisiae pas10* mutants, i.e., catalase (Fig. 10 *C*), SKL-containing proteins (see Fig. *11* compare *A* with *B*) were not present in peroxisomal structures, however, thiolase, which has a PTS2, seemed to accumulate in subcellular structures as judged by immunofluorescence (Fig. 10 *B*) (see also Motley et al., 1994, in which this cell line is referred to as AAL85AD). In contrast, the FAIR-T cell line did not contain either SKL proteins (Fig. 11 *C*) or thiolase (Fig. 10 *E*) in its peroxisomal ghosts. Both cell lines, however, showed a comparable but clearly aberrant immunofluorescence staining with anti-PMP70 (Fig. 10, *A* and *D*).

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 pJ7Ω 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

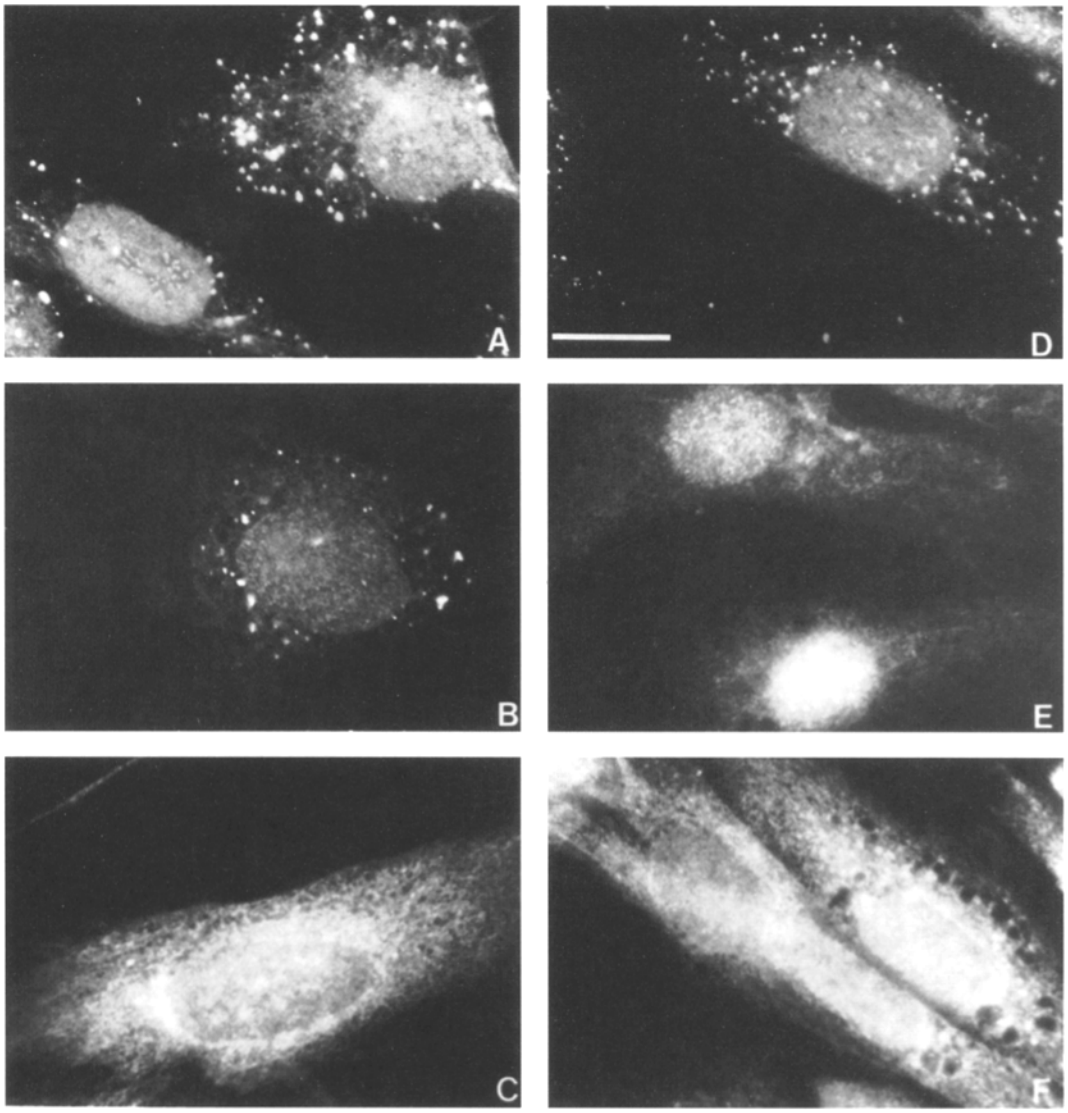

Figure 10. Presence and subcellular localization of PMP70, 3-keto-acyl-Coenzyme A thiolase, and catalase in the cultured skin fibroblasts from group two peroxisomal disorders. Skin fibroblasts from an nALD patient ALA-T (*A–C*) and a ZS patient FAIR-T (*D–F*), belonging to complementation group two, were processed for immunofluorescence and stained with anti-PMP70 (*A* and *D*), anti-thiolase (*B* and *E*) and anti-catalase (*C* and *F*). Bar, 20 μm.

complements the *P. pastoris pas8* mutant for growth on oleate (Table I). 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).

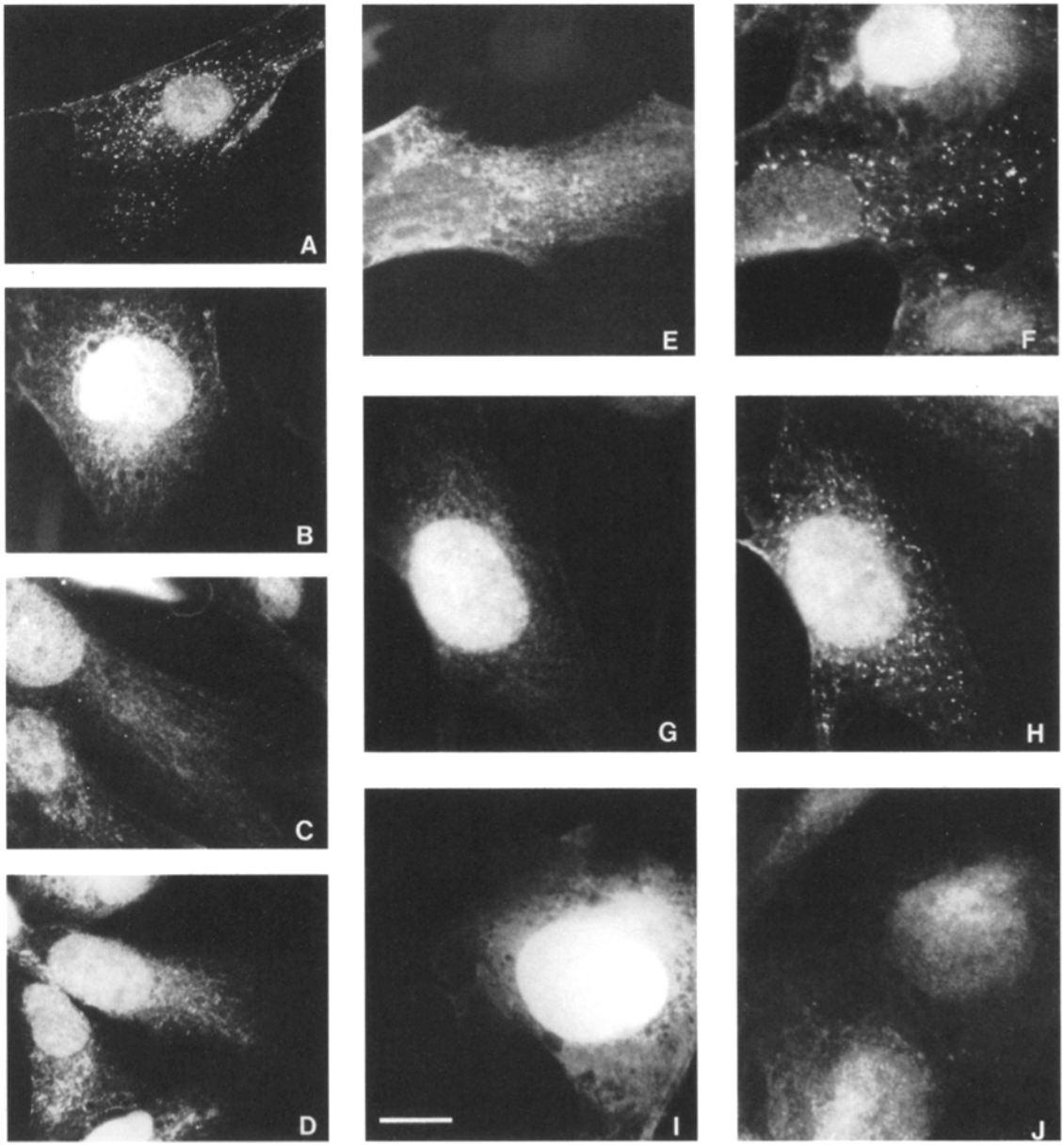

Figure 11. PTS1R restores the ability to import SKL-proteins in cultured skin fibroblasts from group two patients with disorders of peroxisome biogenesis. Skin fibroblasts from a control subject PUCK (*A*), an nALD patient ALA-T (*B*) and two ZS patients FAIR-T (*C*) and GM4340-T (*D*), were processed for immunofluorescence and stained with anti-SKL antibody. A full-length PTS1R expression construct was microinjected into the nuclei of ALA-T, FAIR-T, and GM4340-T cells along with a guinea pig IgG to distinguish injected cells from noninjected cells. (*E* and *F*) ALA-T fibroblasts (complementation group two) that were processed for double-labeling immunofluorescence; *E* shows a microinjected cell stained with anti–guinea pig Ig FITC, *F* shows the corresponding image stained with rabbit anti-SKL and anti–rabbit Ig rhodamine. (*G* and *H*) FAIR-T fibroblasts (complementation group two) and (*I* and *J*) GM4340-T fibroblasts (complementation group four) stained as in *E* and *F*. Bar, 20 μm.

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.,

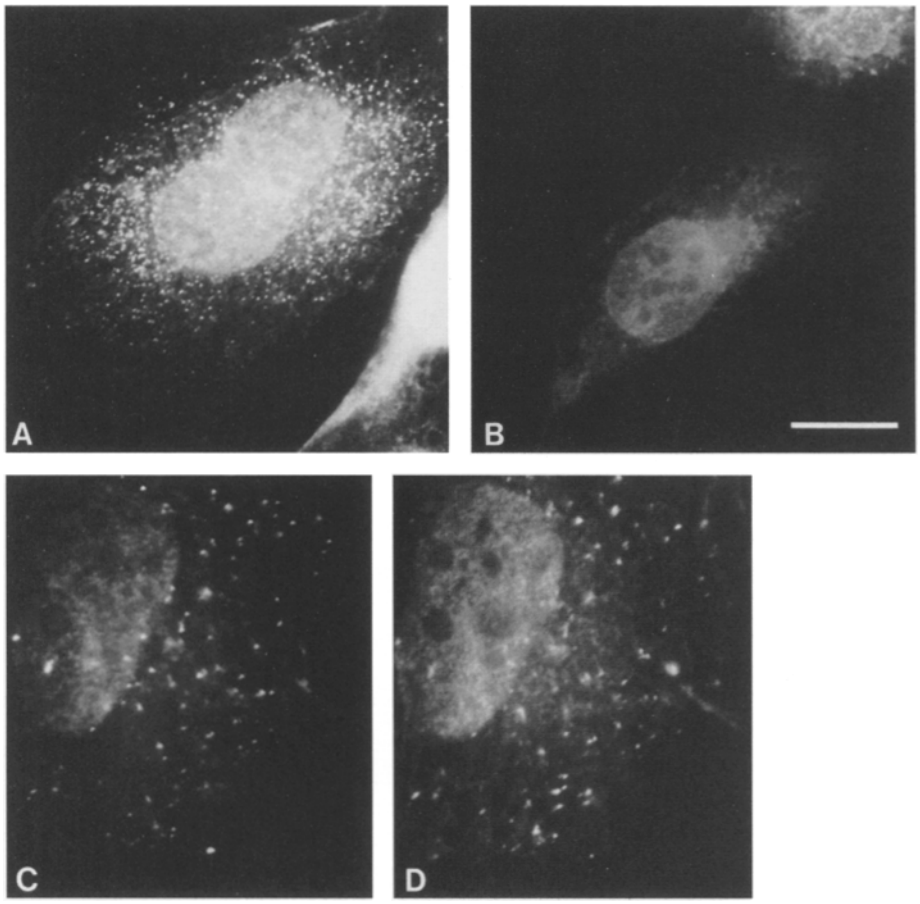

Figure 12. SKL-containing proteins are imported into the peroxisomal matrix when cultured skin fibroblasts from group two peroxisomal disorders are complemented with PTS1R. Skin fibroblasts from an nALD patient, ALA-T as a representative of complementation group two were transfected with a full-length 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) (7q11.12-q11.23) 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 waddler (*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 (McCollum 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).

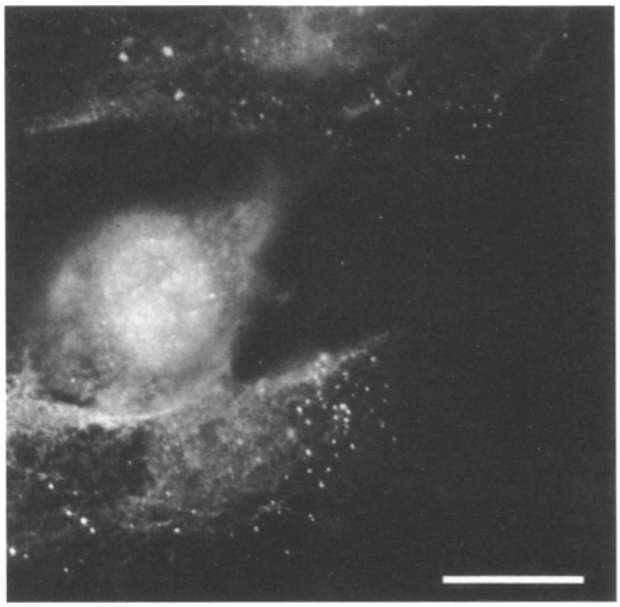

Figure 13. Complementation of FAIR-T cells with PTS1R also restores import of PTS2-containing proteins. Skin fibroblasts from a ZS patient FAIR-T, belonging to complementation group two, were transfected with a full-length PTS1R expression construct. After 48 h the cells were processed for immunofluorescence and stained with anti-thiolase. Bar, 20 μm.

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) affected in two other complementation groups of ZS patients are known (Gartner et al., 1992; Shimozawa et al., 1992), nothing is known about the functions of these proteins. 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 understood. This study also represents one of the few examples 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 antiserum, 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 antiserum, to Stanley Terlecky for the preparation of the ΔSKL 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 results.

This study was supported by Human Frontier Science Program Organization (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 HG00298 (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. Subramani.

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_h_4, and 953_f_12. Given the STS content information for these YACs, we conclude that PTS1R maps near genes for the complement component Clr and for CD4 (Krauter, K., personal communication).

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

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