Multiple PEX Genes Are Required for Proper Subcellular Distribution and Stability of Pex5p, the PTS1 Receptor: Evidence That PTS1 Protein Import Is Mediated by a Cycling Receptor

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Abstract. PEX5 encodes the type-1 peroxisomal targeting signal (PTS1) receptor, one of at least 15 peroxins required for peroxisome biogenesis. Pex5p has a bimodal distribution within the cell, mostly cytosolic with a small amount bound to peroxisomes. This distribution indicates that Pex5p may function as a cycling receptor, a mode of action likely to require interaction with additional peroxins. Loss of peroxins required for protein translocation into the peroxisome (PEX2 or PEX12) resulted in accumulation of Pex5p at docking sites on the peroxisome surface. Pex5p also accumulated on peroxisomes in normal cells under conditions which inhibit protein translocation into peroxisomes (low temperature or ATP depletion), returned to the cytoplasm when translocation was restored, and reaccumulated on peroxisomes when translocation was again inhibited. Translocation inhibiting conditions did not result in Pex5p redistribution in cells that lack detectable peroxisomes. Thus, it appears that Pex5p can cycle repeatedly between the cytoplasm and peroxisome. Altered activity of the peroxin defective in CG7 cells leads to accumulation of Pex5p within the peroxisome, indicating that Pex5p may actually enter the peroxisome lumen at one point in its cycle. In addition, we found that the PTS1 receptor was extremely unstable in the peroxin-deficient CG1, CG4, and CG8 cells. Altered distribution or stability of the PTS1 receptor in all cells with a defect in PTS1 protein import implies that the genes mutated in these cell lines encode proteins with a direct role in peroxisomal protein import.

PEROXISOMAL proteins are encoded by nuclear genes, synthesized in the cytoplasm, and imported into peroxisomes posttranslationally. Therefore, the cellular mechanisms which mediate import of newly synthesized peroxisomal proteins must be able to recognize these proteins, transport them from the cytoplasm to the peroxisome, and then catalyze their translocation across the peroxisome membrane. Recognition of peroxisomal proteins is dependent upon the presence of peroxisomal targeting signals (PTSs)[1] within these proteins. Most peroxisomal matrix proteins contain the PTS1, a tripeptide consisting of serine-lysine-leucine$_{COOH}$, or a conservative variant (Gould et al., 1989; de Hoop and AB, 1992). In contrast to the PTS1, the PTS2 is located at the NH_2 terminus of proteins and mediates import of far fewer proteins (Osumi et al., 1991; Swinkels et al., 1991; Subramani, 1993). Like the PTS1, the PTS2 also appears to fit a loose consensus sequence, R/KQ/Hx$_5$Q/HL, and also directs proteins to the peroxisome lumen. Evidence for internal PTSs has also been reported (Kragler et al., 1993; Small et al., 1988), but the critical features of these signals are yet to be defined. Interestingly, proteins can enter the peroxisome lumen even if they lack a PTS, provided that they oligomerize with a PTS-containing protein before import (Glover et al., 1994; McNew and Goodman, 1994). Thus, it is possible that homo- or hetero-oligomerization domains may function as targeting signals even though they do not mediate direct interaction with a PTS receptor. Another surprising aspect of peroxisomal protein import is that integral peroxisomal membrane proteins (IPMPs) do not simply use a matrix targeting signal in combination with a hydrophobic membrane-spanning segment (Subramani, 1993). Instead, they appear to contain a completely distinct type of targeting signal (Dyer et al., 1996) and an insertion element which remains to be identified.

Many recent advances in understanding peroxisome biogenesis have come from the analysis of mutant cells defective in peroxisomal protein import. Human cells with this phenotype have been obtained from patients afflicted with the peroxisome biogenesis disorders (PBD) (Lazarow and Moser, 1995; Moser et al., 1995; Motley et al.,

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1. *Abbreviations used in this paper*: IPMP, integral peroxisomal membrane protein; PBD, peroxisome biogenesis disorder; *pex*, peroxisome assembly; PTS, peroxisomal targeting signal; TPR, tetratricopeptide repeat.

1994; Santos et al., 1988; Slawecki et al., 1995; Walton et al., 1992). The PBD are a group of lethal genetic diseases including Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and rhizomelic chondrodysplasia punctata. Of these, Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease represent gradations of the same phenotypes (Zellweger syndrome being the most severe), while rhizomelic chondrodysplasia punctata is characterized by a very different set of characteristics. Although these diseases span a wide range of clinical phenotypes, all are characterized by severe mental retardation and appear to be the result of specific defects in peroxisome biogenesis. Somatic cell fusion studies using skin fibroblasts from PBD patients indicate that defects in any of at least ten genes can cause these disorders (Moser et al., 1995; Shimozawa et al., 1993). The analysis of the protein import capacities of PBD cell lines from groups 1-4 and 7-11 (Slawecki et al., 1995) has revealed that defects in all but the CG11 gene can result in defects in both PTS1 and PTS2 protein import. Furthermore, while PBD cell lines representing CGs 1, 4, and 8 retain the ability to import at least some PTS1 and PTS2 proteins into peroxisomes, some CG3 and CG10 cells exhibit a complete defect in matrix protein import (Slawecki et al., 1995). CG2 patients have been shown to carry mutations in PEX5, the PTS1 receptor gene, and exhibit either a specific defect in PTS1 protein import or an inability to import PTS1 and PTS2 proteins (Dodt et al., 1995; Wiemer et al., 1995). Not surprisingly, the selective PTS2 protein import defect of CG11 cells (Motley et al., 1994; Slawecki et al., 1995) is caused by mutations in the PTS2 receptor gene, PEX7 (Braverman, N., G. Dodt, S.J. Gould, and D. Valle, manuscript submitted for publication). CG9 cells are unique in that they lack detectable peroxisomes and are likely to be defective in either synthesis of peroxisome membranes or targeting of integral peroxisomal membrane proteins (Chang C.-C., G. Dodt, and S.J. Gould, manuscript in preparation). Other known PBD genes include PEX2, PEX6, and PEX12, the genes defective in complementation groups 10, 4, and 3, respectively (Shimozawa et al., 1992; Yahraus et al., 1996; Chang, C.-C., D. Valle, and S. J. Gould, manuscript in preparation).

Yeast strains unable to import the normal complement of peroxisomal proteins have also been isolated. These peroxisome assembly (*pex*) mutants have been isolated using a variety of genetic screens and selections in several different organisms: *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, and *Yarrowia lipolytica* (Cregg et al., 1990; Elgersma et al., 1993; Erdmann et al., 1989; Gould et al., 1992; Liu et al., 1992; Nuttley et al., 1993; Kalish et al., 1996). To date, fifteen distinct complementation groups of yeast *pex* mutants have been reported (Elgersma et al., 1993) and 13 PEX proteins, or peroxins, have been characterized (Distel et al., 1996). As with human PBD cells, yeast *pex* mutants appear to fall within five general categories: (*1*) those with a complete defect in protein translocation (Kalish et al., 1995), (*2*) those with reduced import efficiency (Heyman et al., 1994; Spong and Subramani, 1993), (*3*) a single complementation group that exhibits a PTS1-specific import defect (McCollum et al., 1993; van der Klei et al., 1993, 1995), (*4*) another with a PTS2-specific import defect (Marzioch et al., 1994; Zhang and Lazarow, 1995), and (*5*) one with a defect in peroxisome synthesis and/or targeting of IPMPs (Waterham et al., 1993; Baerends et al., 1996; Wiemer et al., 1996).

The PTS1-specific import defect in both human and yeast cells is caused by mutations in *PEX5*, the PTS1 receptor gene. The human and yeast forms of the PTS1 receptor are closely related tetratricopeptide repeat (TPR) proteins with intrinsic PTS1-binding activity mediated via their TPR domains (Dodt et al., 1995; Terlecky et al., 1995). However, the yeast and human forms of Pex5p differ because the loss of the yeast PTS1 receptor has no effect on the import of PT2 proteins (McCollum et al., 1993; van der Leij et al., 1993), while the human PTS1 receptor is required for PTS2 protein import (Dodt et al., 1995; Slawecki et al., 1995; Wiemer et al., 1995; Braverman, N., G. Dodt, S.J. Gould, and D. Valle, manuscript submitted for publication).

Because the PTS1 receptor plays such a central role in the protein import process, knowing its subcellular distribution would help distinguish between several possible models for peroxisomal protein import. Although early reports on the distribution of the PTS1 receptor from the yeast *P. pastoris* (PpPex5p) indicated it was an integral peroxisomal membrane protein (McCollum et al., 1993; Terlecky et al., 1995), we have found that PpPex5p is a predominantly cytoplasmic protein: only 5% or less is peroxisome associated at steady state (Gould et al., 1996). This distribution closely mirrors that reported for the human PTS1 receptor (Dodt et al., 1995), as well as that of Pex5p from the yeast *S. cerevisiae* (Elgersma et al., 1996).

The bimodal distribution of Pex5p indicates that the receptor may cycle between these two compartments as it mediates transport of newly synthesized PTS1 proteins to and into the peroxisome. The recent identification of a docking factor for the PTS1 receptor in the peroxisome membrane lends further support to this notion (Gould et al., 1996; Elgersma et al., 1996; Erdmann and Blobel, 1996). However, there is no other evidence for the cycling receptor model. Elucidation of many biochemical processes has relied on the characterization of precursors which accumulate in cells blocked in different steps of a given pathway. We applied this principle of precursor accumulation to the analysis of PTS1 receptor function by examining the distribution and stability of Pex5p in cells lacking other defined and undefined peroxins blocked at different steps in peroxisomal protein import. Our data indicate that Pex5p is a mobile receptor capable of cycling repeatedly between the cytoplasm and peroxisome. Furthermore, we observed that multiple peroxins are required for Pex5p stability and that all peroxins required for normal PTS1 protein import affect PTS1 receptor distribution or stability.

Materials and Methods

Cell Lines, Antibodies, and Immunofluorescence

Skin fibroblasts from PBD patients are referred to by their PBD number and were cultured as described (Slawecki et al., 1995). Rabbit polyclonal antibodies generated against Pex5p have been described previously (Dodt et al., 1995). The polyclonal anti-PMP70 antibodies were provided by S. Subramani, UC San Diego, La Jolla, CA. Monoclonal anti-myc antibodies (1-9E10; BABCO, Berkeley, CA), sheep anti-catalase (The Binding Site, Birmingham, UK), and fluorescent-labeled secondary antibodies (Kirke-

gaard and Perry Laboratories, Gaithersburg, MD) were obtained from commercial sources. Transfection experiments and indirect immunofluorescence microscopy were performed according to established methods (Slawecki et al., 1995). Unless otherwise noted, cells were permeabilized with 1% Triton X-100 for 5 min. For differential permeabilization experiments in which only the plasma membrane is disrupted, cells were incubated in buffered 25 μg/ml digitonin for 5 min instead of 1% Triton X-100 (Swinkels et al., 1991). All differential permeabilization experiments were performed side by side with appropriate controls to ensure that the digitonin treatment did not permeabilize the peroxisome membrane.

ATP Depletion Experiments

Fibroblasts were incubated in either serum-free normal medium DMEM or ATP depletion medium (DMEM lacking glucose, supplemented with 50 mM 2-deoxy D-glucose and 5 mM sodium azide) for specified periods of time. When ATP depletion was combined with low temperature incubation, the cells were first incubated in ATP depletion medium for 15 min at 37°C, and then shifted to 16°C for the indicated period of time.

Metabolic Labeling and Immunoprecipitations

Fibroblasts were grown on 60-mm dishes to 70% confluency in DMEM supplemented with 10% FCS (DMEM/10% FCS). The cells were washed twice with DMEM/5% FCS without methionine and cysteine and incubated in this medium for 30 min. Cells were pulsed for 1 h with 1 mCi [^{35}S]methionine/[^{35}S]cysteine in 1 ml medium. The cells were washed two times with HBSS and chased in DMEM/10%FCS with 10 mM methionine and cysteine for the times indicated and under the stated conditions. After two washes with HBSS the cells were incubated for 15 min on ice in 0.5 ml cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS, 0.1 mM PMSF, and 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Cell lysates were scraped from the plate with an additional 200 μl buffer, agitated vigorously, and cleared by centrifugation at 12,000 *g* for 15 min at 4°C. The resulting supernatant was incubated with 20 μl of a 1:1 slurry of protein A beads (Pharmacia LKB Biotechnology, Piscataway, NJ) for 1 h, the beads were removed by centrifugation, and the resulting supernatant was incubated with 2 ml of either preimmune or immune anti-Pex5p serum for 2 h at 4°C. 20 μl of protein A beads were added, incubated for an additional hour, and collected by centrifugation. The beads were washed and the proteins recovered in 40 μl SDS sample buffer. Samples were boiled for 5 min, resolved by SDS-PAGE, and labeled proteins were detected by fluorography.

Protein Extracts and Immunoblotting

For determining levels of Pex5p in the cell, fibroblasts were grown to 90% confluency in 60-mm dishes, washed two times with HBSS, removed from the plate, and recovered by centrifugation at 500 *g* for 10 min. After two additional washes, the cells were resuspended in 50 μl water. An aliquot was removed for protein determination and the samples were immediately boiled in SDS sample buffer and frozen. For cell fractionations, cells were lysed as described (Dodt et al., 1995) and organelles were separated from cytosol by centrifugation at 14,000 *g*. Immunoblot analysis of the supernatant and pellet fractions using anti-PMP70 antibodies confirmed that the 14,000-*g* spin was sufficient to pellet all of the peroxisome membranes in the cell. The quantity of Pex5p in each supernatant and pellet sample was determined by SDS-PAGE and immunoblot using anti-Pex5p antibodies (Dodt et al., 1995).

Results

Pex5p Accumulates on Peroxisomes Under Translocation Inhibiting Conditions

The observation that the PTS1 receptor in both human and yeast cells is a predominantly cytoplasmic protein (Dodt et al., 1995; Gould et al., 1996) indicated that Pex5p may function as a cycling receptor, guiding newly synthesized peroxisomal proteins from the cytoplasm to the peroxisome and then returning to the cytoplasm to repeat the process. Since a cycling receptor would have to migrate to and bind the peroxisome before the protein translocation event, inhibiting translocation would lead to Pex5p accumulation at the prior step in import, i.e., docking to the peroxisome membrane. The extent of accumulation would depend on the relative abundance of the PTS1 receptor and Pex13p, the IPMP which functions as a docking factor for the receptor (Gould et al., 1996; Elgersma et al., 1996; Erdmann and Blobel, 1996), as well as the degree of translocation inhibition.

Table I. The Peroxisomal Protein Import Defects of PBD Cell Lines Representing Nine Complementation Groups

Complementation group	PTS1-specific import defect	PTS2-specific import defect	Complete PTS1 and PTS2 protein import defect	Partial PTS1 and PTS2 protein import defect
CG1	0	0	0	10 of 10
CG2	2 of 3	0	1 of 3	0
CG3	0	0	4 of 4	0
CG4	0	0	0	20 of 20
CG7	0	0	0	3 of 3
CG8	0	0	0	8 of 8
CG9	0	0	1 of 1	0
CG10	0	0	2 of 2	0
CG11	0	13 of 13	0	0

The cycling receptor hypothesis was first tested by comparing the distribution of Pex5p in control fibroblasts and fibroblasts that are unable to import matrix proteins into the peroxisome lumen. As previously reported (Dodt et al., 1995), the amount of peroxisome associated Pex5p in normal human fibroblasts is so low that only the cytoplasmic form is detected using our standard indirect immunofluorescence protocol (Fig. 1 *A*). In contrast, peroxisome associated Pex5p was readily detected in fibroblasts from both CG3 (Fig. 1 *B*) and CG10 (Fig. 1 *C*) PBD patients, cells which are unable to translocate peroxisomal matrix proteins into the peroxisome (Table I; Slawecki et al., 1995). The colocalization of Pex5p and the peroxisomal membrane marker PMP70myc in these cells (Fig. 1 *D*) confirmed that the Pex5p-containing vesicles were indeed peroxisomes (not all peroxisomes contained both Pex5p and PMP70myc but heterogeneity of peroxisomal content has been noted previously; Luers et al., 1993). To test whether Pex5p had accumulated on the outer surface of peroxisomes or had been imported into these vesicles, CG3 and CG10 cells were subjected to differential permeabilization immunofluorescence experiments in which only cytoplasmically exposed antigens are detected (Swinkels et al., 1991). Peroxisomal Pex5p was still detected under these conditions, indicating that it had accumulated on or near the outer surface of the peroxisome in CG3 and CG10 cells (Fig. 1, *E* and *F*).

The subcellular distribution of Pex5p in normal and CG10 fibroblasts was also examined by differential centrifugation. Cell free lysates were prepared from N1 (unaffected control) and CG10 fibroblasts and separated into supernatant and organelle fractions by centrifugation at 14,000 *g* (it was not possible to further purify peroxisomes from these cells because they migrate at an anomalously low density due to their lack of matrix content). Each sample was then subjected to immunoblot analysis using antibodies specific for Pex5p (Fig. 2). As demonstrated previously (Dodt et al., 1995), only a small amount (5%) of

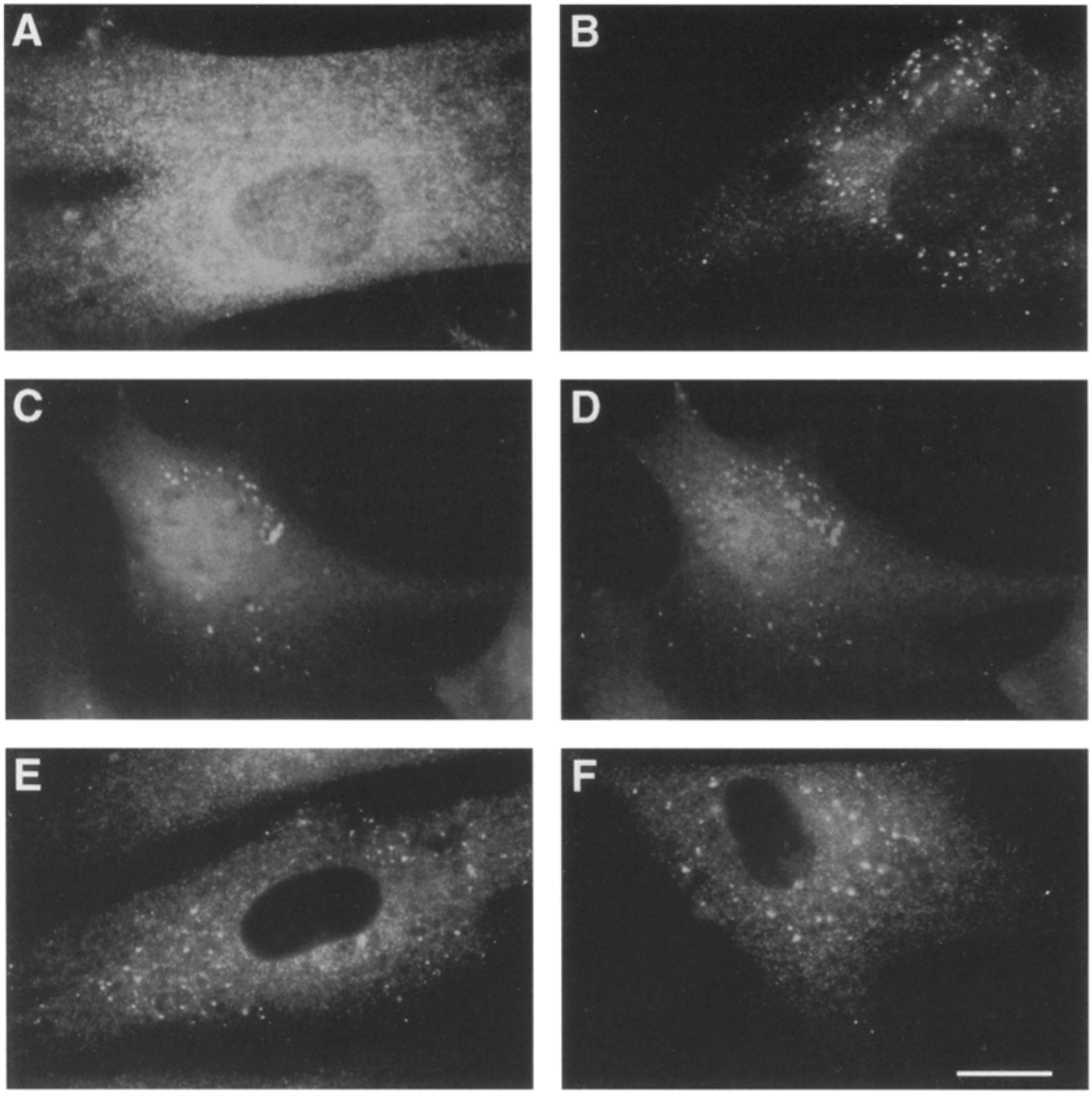

Figure 1. Pex5p accumulates on the peroxisome surface in PBD cells defective in peroxisomal protein translocation. Indirect immunofluorescence microscopy using antibodies specific for Pex5p in (*A*) normal human fibroblasts and (*B*) CG3 fibroblasts (PBD006). The CG10 cell line PBD094 was transfected with a vector designed to express PMP70myc, allowing detection of peroxisome membranes, and subsequently processed for indirect immunofluorescence using antibodies specific for (*C*) Pex5p and (*D*) the myc epitope tag at the COOH-terminus of PMP70myc. (*E*) CG3 (PBD095) and (*F*) CG10 (PBD062) cells were also processed for differential (digitonin) permeabilization indirect immunofluorescence using anti-Pex5p antibodies. Bar, 25 μm.

Pex5p was organelle associated in wild-type cells. In contrast, 25% of Pex5p was organelle associated in CG10 cells, confirming that the translocation block correlated with a redistribution of Pex5p from the cytoplasm to the peroxisome. It is important to note that a significant amount of Pex5p is cytosolic even in CG10 cells. This is not surprising considering that Pex13p, the docking factor for the PTS1 receptor, is a low abundance peroxisomal membrane protein (our unpublished observations), ~100-fold less abundant than PMP27, another peroxisomal membrane protein (Erdmann, R., personal communication). Serial analysis of gene expression (Velculescu et al., 1995) in human cells revealed that PEX5 mRNA may be fourfold more abundant than PEX13 mRNA in human cells (data not shown). If the levels of Pex5p and Pex13p reflect the abundance of their mRNAs, 25% would be the most Pex5p that could accumulate on peroxisomes.

We next examined whether the PTS1 receptor could be trapped on the peroxisome in normal human fibroblasts by incubating the cells at low temperature or under ATP depleting conditions. Previous studies have demonstrated that protein translocation into the peroxisome is inhibited by low temperature or by depleting ATP levels (Imanaka et al., 1987; Miyazawa et al., 1989; Walton et al., 1992; Wendland and Subramani, 1993). Pex5p is predominantly cytoplasmic in normal human fibroblasts (Fig. 3 *A*) but accumulated on peroxisomes after a 2-h incubation at 16°C in ATP depletion medium (lacking glucose and supplemented with 2-deoxyglucose and sodium azide) (Fig. 3 *B*). Differential permeabilization and colocalization experi-

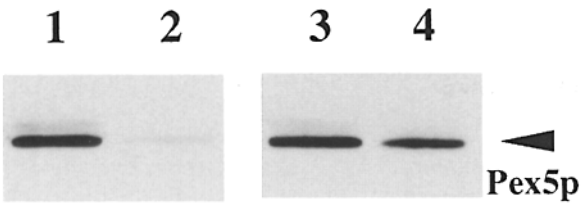

Figure 2. Levels of peroxisome associated Pex5p are elevated fivefold in PEX2-deficient cells. The CG10 cell line PBD094 and the control cell line N1 (Slawecki et al., 1995) were gently lysed and separated into organellar pellet and cytosolic supernatant fractions by centrifugation. The supernatant and pellet fractions from each cell line were separated by SDS-PAGE and processed for immunoblot with anti-Pex5p antibodies. Lane *1*, control supernatant; lane *2*, control pellet; lane *3*, CG10 supernatant; lane *4*, CG10 pellet. The slower migrating band represents a modified form of Pex5p. The precise nature of the modification remains to be determined. Immunoblot analysis of the same samples with anti-PMP70 antibodies revealed that the 14,000-*g* spin was sufficient to quantitatively pellet peroxisomes from the lysates.

ments confirmed that Pex5p was present on the outer surface of the peroxisome under these conditions (data not shown).

The distribution of Pex5p in these experiments was also examined by differential centrifugation. Normal human fibroblasts incubated in growth medium or in ATP depletion medium for 1 h were gently lysed and the resulting extract was separated into a supernatant of cytosolic proteins and an organelle pellet. Immunoblot analysis of these fractions using anti-Pex5p antibodies (Fig. 4) revealed that 14% of Pex5p was organelle (peroxisome) associated under translocation inhibiting conditions. This is a significant increase relative to the 5% observed in cells grown under normal conditions but somewhat less than the 25% accumulation observed in CG10 cells (refer to Fig. 2). Possible reasons for the lower accumulation of peroxisome associated Pex5p in this experiment are that (*1*) ATP depletion may only inhibit translocation, not abolish the process, (*2*) the technique used to deplete ATP is unlikely to eliminate all ATP in the cell, and (*3*) the duration of the translocation inhibition in these experiments was only 1 h, as compared to constitutive inhibition in the translocation defective CG10 cells.

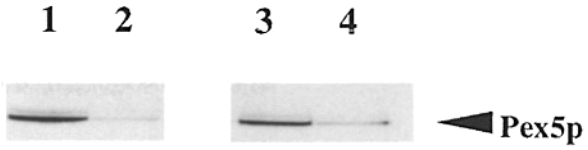

Figure 4. Levels of peroxisome associated Pex5p in normal cells increase under translocation inhibiting conditions. The control cell line N1 was grown under normal conditions or in ATP depletion medium for 1 h and subsequently lysed. Each lysate was separated into organellar pellet and cytosolic supernatant fractions by centrifugation. The supernatant and pellet fractions from both samples were separated by SDS-PAGE and processed for immunoblot with anti-Pex5p antibodies. Lane *1*, control supernatant; lane *2*, control pellet; lane *3*, -ATP supernatant; lane *4*, -ATP pellet.

Pex5p Is a Cycling Receptor

If the migration of cytoplasmic Pex5p to the peroxisome represented the first half of a normal transport circuit for Pex5p, then releasing the translocation inhibition should allow the receptor to continue its circuit and return to the cytoplasm. This hypothesis was tested in normal cells in which translocation was inhibited by low temperature alone. Pex5p accumulated on peroxisomes after a 2-h incubation at 16°C (Fig. 5, *A* and *B*) but returned to the cytoplasm after the cells were shifted back to 37°C for 1 h (Fig. 5 *C*). When the inhibited and recovered cells (2 h at 16°C followed by 1 h at 37°C) were shifted back to 16°C for an additional 2 h, Pex5p again accumulated on peroxisomes (Fig. 5 *D*), indicating that the receptor was capable of repeatedly cycling between the cytoplasm and peroxisome.

Although these results were consistent with a model in which the PTS1 receptor cycles between the cytoplasm and peroxisome, these data were also consistent with selective degradation of the cytoplasmic form of the PTS1 receptor at 16°C, with rapid resynthesis at 37°C. To address this possibility, normal human fibroblasts were pulse labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 1 h at 37°C. After the pulse, cells were lysed immediately (t = 0), chased for 5 h at 37°C (5 h 37°C), or chased for 2 h at 16°C, 1 h at 37°C, and 2 h at 16°C (5 h TS (temperature shift)). At each time point the cells were lysed

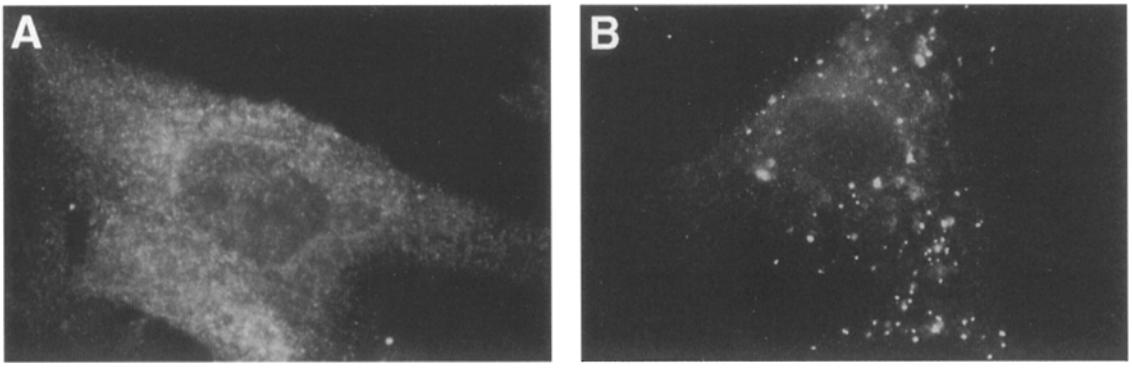

Figure 3. Conditions which inhibit protein translocation across the peroxisome membrane lead to accumulation of Pex5p on peroxisomes. The distribution of Pex5p in normal skin fibroblasts incubated at (*A*) 37°C in normal growth medium or (*B*) for 1 h at 16°C in ATP depletion medium was determined by indirect immunofluorescence microscopy. Bar, 25 μm.

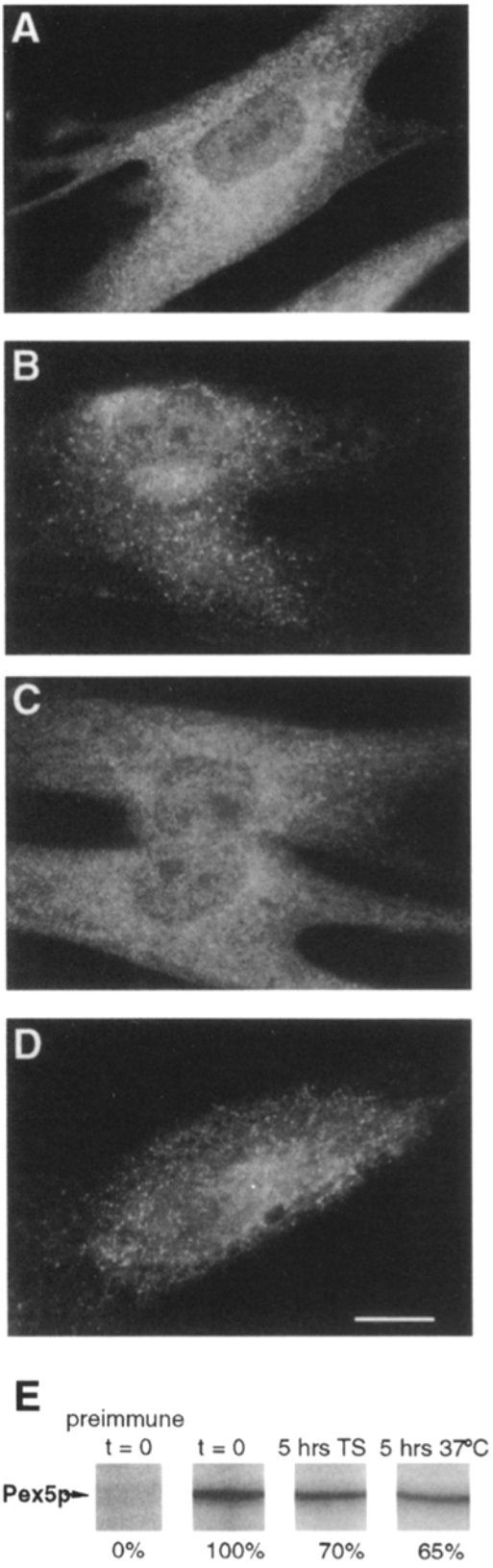

Figure 5. Peroxisomal accumulation of Pex5p in response to low temperature incubation is reversible. Normal human fibroblasts were processed for indirect immunofluorescence after (*A*) incubation at 37°C, (*B*) a 2-h incubation at 16°C, (*C*) a 2-h incubation at 16°C followed by a 1-h incubation at 37°C, and (*D*) incubation for 2 h at 16°C, 1 h at 37°C, followed by an additional 2 h at 16°C. (*E*) Pulse-chase analysis of Pex5p during the temperature shift experiment. Cells were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine, washed, and either lysed immediately or chased for 5 h under the identical temperature shift conditions as in *D* above (5 h TS) or for 5 h at 37°C (5 h 37°C). Pex5p was immunoprecipitated from the lysates at the given time points. 70% of Pex5p synthesized at the beginning of the experiment was still present at its conclusion whereas slightly less (65%) was present in cells incubated at 37°C for the same length of time. Bar, (*D*) 25 μm.

and Pex5p was immediately immunoprecipitated from the lysate. Quantitation of Pex5p levels in each sample (Fig. 5 *E*) revealed that the temperature shift conditions resulted in slight stabilization of the PTS1 receptor. Thus, there was no evidence for selective loss of cytoplasmic Pex5p by incubation at 16°C. Furthermore, over the 5 h course of the experiment almost 70% of Pex5p present at $t = 0$ was present at $t = 5$ h, indicating that we were primarily observing the same population of PTS1 receptor molecules throughout the experiment. Similar changes in Pex5p distribution were observed when this experiment was repeated in the presence of cycloheximide (data not shown).

We also tested whether Pex5p would cycle back to the cytoplasm in CG10 cells after correction of their translocation defect. CG10 cells are homozygous for inactivating mutations in the PEX2 gene and transfection of the PEX2 cDNA into CG10 cells restores peroxisomal protein import (Shimozawa et al., 1992). We transfected the CG10 cell line PBD094 with a plasmid which expresses the wild-type PEX2 gene. Only cytoplasmic Pex5p was detected in CG10 cells expressing PEX2 while noncomplemented CG10 cells retained the peroxisomal staining pattern for Pex5p (data not shown).

Pex5p Cannot be Trapped on or in Peroxisomes in Cells Which Lack Peroxisome Membranes

We recently completed an analysis of IPMP import in PBD cell lines (Chang, C.-C., G. Dodt, and S.J. Gould, manuscript in preparation) to complement our earlier analysis of matrix protein import in these cells (Slawecki et al., 1995). As expected from the seminal observations of Santos et al. (1988), IPMP targeting was intact in the vast majority of PBD cells, including representatives of complementation groups 1, 2, 3, 4, 7, 8, 10, and 11. However, peroxisomes could not be detected in PBD061 cells (the sole representative of PBD group 9), indicating that the CG9 gene is required for synthesis of peroxisome membranes or targeting IPMPs into the peroxisome membrane. The absence of detectable peroxisome membranes in PBD061 cells makes them an excellent cell line in which to test the specificity of Pex5p accumulation under translocation inhibiting conditions. Only cytoplasmic Pex5p was detected in PBD061 cells under normal growth conditions (Fig. 6 *A*) or after incubation in ATP depleting medium at 16°C for 1 h (Fig. 6 *B*), conditions which lead to significant peroxisomal accumulation of Pex5p in control cells.

The PTS1 Receptor Is Translocated into Peroxisomes in CG7 Cells

In contrast to CG3, CG10, and CG9 cells, all three CG7 cell lines import detectable levels of both PTS1 and PTS2

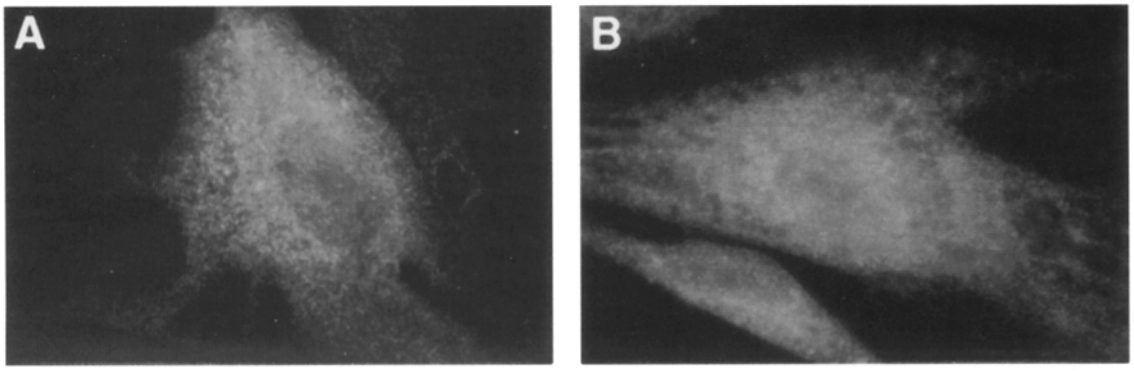

Figure 6. Pex5p cannot be trapped on peroxisomes in cells which lack detectable peroxisomes. The sole CG9 cell line exhibits a defect in synthesis of peroxisome membranes. The distribution of Pex5p was determined by indirect immunofluorescence in PBD061 (CG9) cells (*A*) incubated at 37°C in normal growth medium and (*B*) incubated for 1 h at 16°C in ATP depletion medium. Bar, 25 μm.

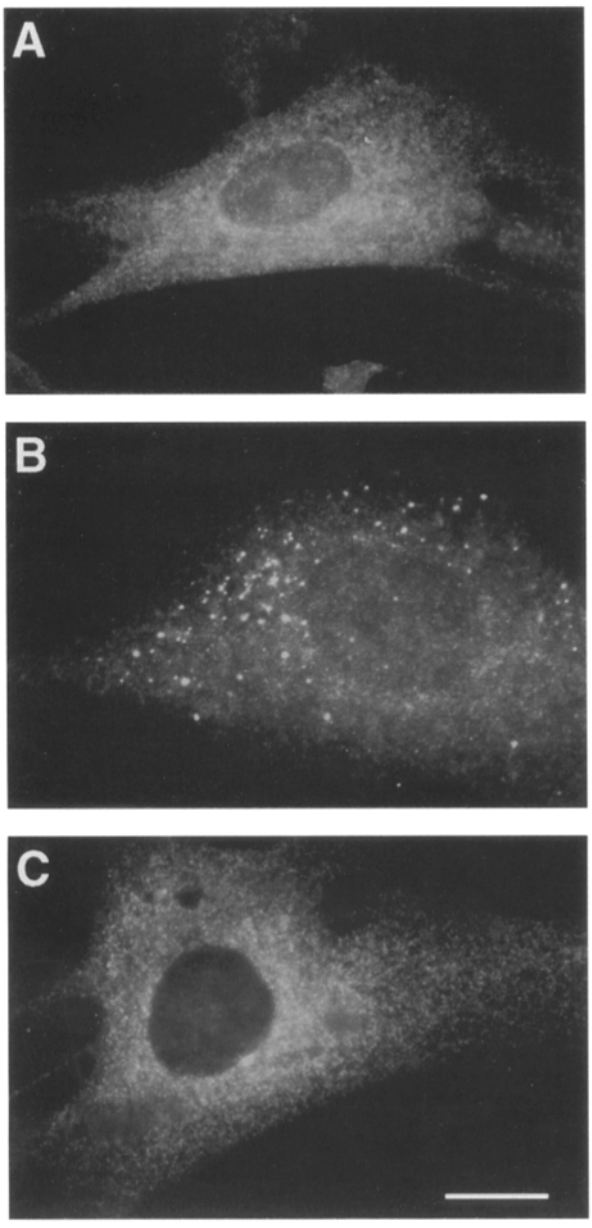

Figure 7. Pex5p is translocated into the peroxisome in CG7 cells. The distribution of Pex5p was determined by indirect immunofluorescence using our standard protocol in (*A*) normal fibroblasts and (*B*) the CG7 cell line PBD054. (*C*) Differential (digitonin) permeabilization of just the plasma membrane (digitonin permeabilization) rendered the peroxisome associated Pex5p of PBD054 cells undetectable in indirect immunofluorescence experiments. Bar, 25 μm.

proteins into peroxisomes. In fact, the amount of matrix protein import in these cell lines was considerably higher than in any other PBD cell lines we have examined (Table I; Slawecki et al., 1995). Based on their high degree of matrix protein import, we expected that the distribution of the PTS1 receptor in CG7 cells would resemble that of wild-type cells. Surprisingly, Pex5p was peroxisome associated in the CG7 cell lines PBD054 (Fig. 7 *B*), PBD052, and PBD053. Even more surprising was the fact that vesicular Pex5p could not be detected in differential permeabilization immunofluorescence experiments (Fig. 7 *C*), indicating that Pex5p is translocated into the peroxisome lumen in these CG7 cells.

The Stability of the PTS1 Receptor Is Severely Reduced in CG1, CG4, and CG8 Cells

Immunofluorescence analysis failed to detect Pex5p in CG1 cells. To determine whether levels of Pex5p were reduced in these cells, total cell protein was isolated from control and PBD cells and Pex5p abundance was determined by immunoblot (Fig. 8, *A* and *B*; Table II). Each gel contained at least one and often two control samples for standardization. Also, with the exception of CG7 cells which are all from neonatal adrenoleukodystrophy patients, all PBD cells chosen for this analysis were from Zellweger syndrome patients, reducing the possibility that partially defective alleles would skew the results. Relative to control fibroblasts, levels of Pex5p were reduced ~20-fold in each of five different CG1 cells. CG4 cells contained 7–20-fold less Pex5p, and the sole CG8 cell assayed contained only 1/7 the amount of Pex5p as control cells. Interestingly, CG1, 4, and 8 cells all share the ability to import small amounts of both PTS1 and PTS2 proteins (Slawecki et al., 1995), even in PEX6-deficient CG4 cells which lack PEX6 mRNA (Yahraus et al., 1996). In contrast to reduced Pex5p abundance in the group 1, 4, and 8

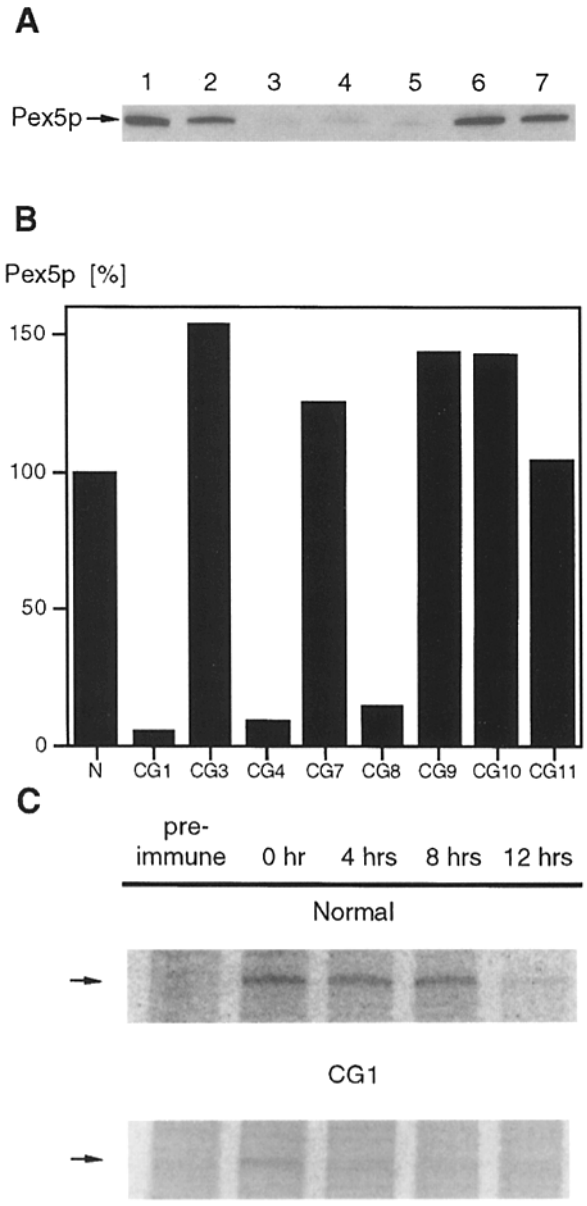

Figure 8. The abundance of Pex5p is severely reduced in PBD cells from complementation groups 1, 4, and 8. (*A*) Immunoblot analysis of Pex5p abundance in PBD and normal human fibroblasts. Equal amounts of total cellular protein from control and PBD cell lines were separated by SDS-PAGE, transferred to membranes, and blotted with anti-Pex5p antibodies. Lane *1*, the CG10 cell line PBD094; lane *2*, the normal fibroblast line N6 (Slawecki et al., 1995); lane *3*, the CG1 cell line PBD002; lane *4*, the CG4 cell line PBD010; lane *5*, the CG4 cell line PBD039; lane *6*, the CG9 cell line PBD061; lane *7*, the normal fibroblast line N7 (Slawecki et al., 1995). (*B*) Levels of Pex5p in fibroblasts from PBD patients and unaffected controls (Table II) were determined by immunoblot of total cellular protein as shown above in *A*. The abundance of Pex5p was plotted by complementation group (CG) as the percent relative to control cell lines. Although Pex5p abundance was determined only once for most cell lines, multiple independent representatives of most groups were assayed. (*C*) The low abundance of Pex5p in CG1 fibroblasts is a function of receptor instability. Control and CG1 (PBD002) fibroblasts were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine, washed, and either lysed immediately (0 h) or chased for 4, 8, and 12 h. The $t = 0$ lysate was subjected to immunoprecipitation with both preimmune and immune sera; all other samples were precipitated with immune sera only. Quantitation of Pex5p abundance in each sample revealed that the half-life for Pex5p was 6 h in control cells and <1 h in CG1 cells.

Table II. Levels of Pex5p in PBD Fibroblasts

Complementation group	Patient ID number	Pex5p (Percent of normal)
CG1	PBD002	4
	PBD009	7
	PBD022	3
	PBD045	5
	PBD047	6
CG2	PBD005	0
	PBD018	90
CG3	PBD006	162
	PBD040	140
	PBD098	162
CG4	PBD010	13
	PBD051	5
	PBD011	14
	PBD039	4
CG7	PBD052	106
	PBD053	157
	PBD054	112
CG8	PBD059	14
CG9	PBD061	144
CG10	PBD094	125
	PBD062	162
CG11	PBD073	95
	PBD082	113

cells, levels of Pex5p were slightly elevated in the translocation deficient CG3 and CG10 cells and the Pex5p-importing CG7 cells. CG11 cells, which carry mutations in the PTS2 receptor gene PEX7, contained normal levels of Pex5p. Receptor levels were remarkably similar in different cells from the same complementation group even though only a single determination of Pex5p levels was made for many of the cell lines studied.

Levels of PEX5 mRNA were approximately equivalent in control and CG1, 4, and 8 cells (data not shown), indicating that the reduced levels of Pex5p in these PBD cells were not mediated by differential PEX5 mRNA synthesis or stability. Consistent with this hypothesis, pulse-chase experiments demonstrated that the half-life for Pex5p was ~6 h in normal cells but reduced to less than 1 h in a CG1 cell line (Fig. 8 *C*). The inability to calculate a more precise half life for Pex5p in CG1 cells was based on (*1*) the amount of Pex5p labeled at $t = 0$, (*2*) the fact that all of the Pex5p present at $t = 0$ was degraded by the 4-h time point, and (*3*) the detection limit of the assay. Nevertheless, a half-life less than 1 h is in good agreement with the half-life of 20 min deduced from the immunoblot data and the 6-h half-life of Pex5p in control cells. We did not perform the necessary controls that would allow us to make any conclusions based on the relative amount of Pex5p labeled in control and CG1 cells during the 1-h pulse. However,

the reduced amount of Pex5p labeled in CG1 cells is consistent with a half-life of a protein that is significantly shorter than the duration of the pulse labeling period. The 1-h half-life of Pex5p in CG4 cells (Yahraus et al., 1996), a sixfold reduction, is also consistent with the 7–20-fold reduction in Pex5p levels in these cells.

Discussion

The PTS1 receptor has been identified in several different species, is required for import of PTS1 proteins into peroxisomes, and exhibits intrinsic PTS1-binding activity (Dodt et al., 1995; McCollum et al., 1993; van der Leij et al., 1993, 1995). Nevertheless, there are many aspects of PTS1 receptor function that are in question. One of the most important issues is whether the PTS1 receptor has a static distribution, lying on the peroxisome surface, or has a dynamic distribution, cycling repeatedly through multiple cellular environments as it transports newly synthesized PTS1 proteins to the peroxisome.

We have demonstrated that the PTS1 receptor in both human cells (Dodt et al., 1995) and the yeast *P. pastoris* (Gould et al., 1996) is a predominantly cytoplasmic protein, with ~5% of the receptor found in association with the peroxisome under steady-state conditions. A bimodal distribution for the receptor has also been observed by Elgersma et al. (1996) in the yeast *S. cerevisiae* and van der Klei et al. (1995) in the yeast *H. polymorpha.* However, several other reports on the yeast and human PTS1 receptor have suggested that this protein is exclusively associated with the peroxisome membrane and may be an integral peroxisomal membrane protein (Fransen et al., 1995; McCollum et al., 1993; Terlecky et al., 1995). Although we have no explanation for this alternative distribution of the mammalian PTS1 receptor reported by Fransen et al. (1995), the localization of the *P. pastoris* PTS1 receptor to just the peroxisome membrane by McCollum et al. (1993) and Terlecky et al. (1995) was apparently due to experimental artifact (Gould et al., 1996). Thus, the preponderance of evidence suggests that the PTS1 receptor is a predominantly cytoplasmic protein. In the present report we have further defined the behavior of the PTS1 receptor by examining how its distribution and abundance is affected by a variety of genetic and environmental conditions which inhibit protein import into the peroxisome.

The observation that 95% of the PTS1 receptor is normally present in the cytoplasm is, by itself, suggestive that that Pex5p functions as a cycling receptor. In this report we examined the possibility that defects in different steps of peroxisomal protein import might alter the distribution and/or abundance of the PTS1 receptor and whether such alterations might confirm or refute the cycling receptor hypothesis. Just as blocking a particular step in a metabolic pathway leads to accumulation of the precursor for that step, blocking any step in the receptor's cycle should lead to accumulation of the receptor at the prior point in the pathway. Currently, the only aspect of peroxisomal protein import that is generally accepted is that proteins are translocated across the peroxisome membrane. In the cycling receptor hypothesis, blocking the translocation step should result in accumulation of the PTS1 receptor at its docking site on the peroxisome surface, the step prior to translocation. Therefore, the relative amount of peroxisome associated Pex5p should increase under these conditions. Our data demonstrate that the PTS1 receptor accumulates on or near the surface of peroxisomes when protein translocation is blocked, either by loss of putative protein translocation factors (PEX2 and PEX12) in PBD group 3 or 10 fibroblasts, or by low temperature incubation and/or ATP depletion in normal fibroblasts. The fact that Pex5p is released from the peroxisome by reversing the translocation inhibiting conditions provides evidence that the receptor can return to the cytoplasm after accumulating on the peroxisome. The reaccumulation of Pex5p on peroxisomes by reapplication of the translocation inhibiting conditions suggests that the receptor may be capable of cycling repeatedly between the cytoplasm and the peroxisome. As expected, no accumulation of peroxisomal Pex5p was detected in PBD061 cells which lack detectable peroxisomal membranes (Chang, C.-C., G. Dodt, and S.J. Gould, manuscript in preparation).

Blocking protein translocation into peroxisomes did not result in accumulation of all PTS1 receptors on the peroxisome membrane. In the translocation defective CG10 cells that contained 5 times as much peroxisome associated receptor peroxisomal Pex5p still represented just 25% of the total receptor molecules in the cell. This may well be caused by greater abundance of PTS1 receptors relative to Pex13p, the docking factor for the PTS1 receptor. Serial analysis of gene expression (Velculescu et al., 1995) revealed that human cells contain four times more PEX5 mRNA than PEX13 mRNA. If the relative abundance of Pex5p and Pex13p mirror the abundance of their respective mRNAs, then the upper limit of Pex5p that could associate with the peroxisome would be 25%. The possibility that Pex13p may limit peroxisomal accumulation of Pex5p is further supported by the observation that Pex13p is a low abundance protein of the peroxisome membrane (Erdmann, R., personal communication; our unpublished observations).

The results of the translocation inhibition experiments support a model (Fig. 9) in which newly synthesized PTS1 proteins are recognized in the cytoplasm by the PTS1 receptor (step 1), with subsequent transport of the receptor-ligand complex to the peroxisome (step 2), followed by docking of the receptor-ligand complex to Pex13p on the peroxisome surface (step 3). The surprising observation that Pex5p was translocated into the peroxisome lumen in CG7 cells suggests two alternatives for the next step in import, either (4a) receptor-ligand dissociation at the translocation pore, followed by ligand translocation into the peroxisome lumen and receptor recycling; or (4b) translocation of the receptor-ligand complex into the peroxisome lumen, followed by dissociation of the complex and export of the receptor from the peroxisome. The first of these models is consistent with a role for the CG7 gene product in dissociation of the receptor-ligand complex while the latter model would predict that the CG7 gene product is involved in exporting the PTS1 receptor from the peroxisome lumen. Although we have no direct evidence for intraperoxisomal Pex5p under steady-state conditions in normal human fibroblasts, the PTS1 receptor in the yeast *H. polymorpha* (HpPex5p) has been detected in the lumen of the peroxisome in wild-type cells (van der Klei et al.,

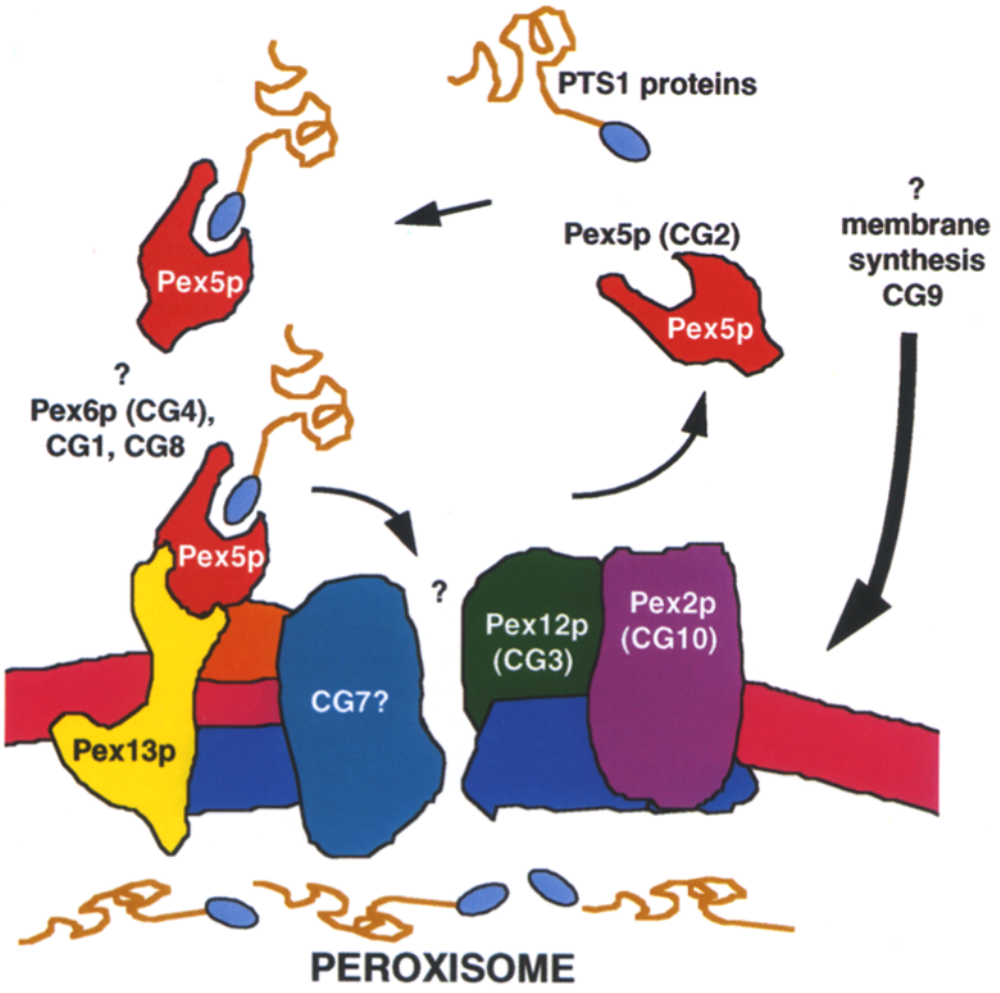

Figure 9. PTS1 protein import is mediated by a cycling PTS1 receptor. The first step in import appears to involve recognition of newly synthesized PTS1 proteins in the cytoplasm by the PTS1 receptor. Next, the receptor-ligand complex is transported to the peroxisome. This process may involve one or more of the peroxins defective in PBD complementation groups 1, 4 (Pex6p), and 8. Binding of this complex to the peroxisome surface appears to be limited, consistent with the existence of a saturable docking factor (Pex13p; Gould et al., 1996). Protein translocation into peroxisomes requires the products of the CG3 (PEX12) and CG10 (PEX2) genes and involves, at a minimum, receptor-ligand dissociation with subsequent ligand translocation. However, translocation of the receptor into peroxisomes in CG7 cell lines raises the possibility that translocation normally occurs by import of the receptor-ligand complex with subsequent dissociation of the complex and receptor export. After delivering its cargo of newly synthesized PTS1 proteins to the peroxisome, the PTS1 receptor returns to the cytoplasm to repeat the cycle. The lack of detectable peroxisomes in CG9 cells suggests that synthesis of peroxisomal membranes and/or targeting of integral peroxisomal membrane proteins are distinct from matrix protein import.

1995), providing direct support for the hypothesis that protein import may normally involve movement of the PTS1 receptor through the peroxisome lumen.

The model for peroxisomal protein import that we have proposed is far from complete. The most obvious omissions are that it does not incorporate the import of PTS2 proteins or IPMPs and does not explain the significance of the pre-import complex of newly synthesized peroxisomal proteins identified by Bellion and Goodman (1987). Furthermore, certain details of the model have yet to be observed. The assumption that the PTS1 receptor binds newly synthesized proteins in the cytoplasm remains to be proven, the implication that Pex2p and Pex12p represent components of the protein translocation pore is based solely on circumstantial evidence, and the implied role of the CG1, 4, and 8 gene products in the early steps in the PTS1 receptor cycle are based primarily on the fact that the phenotypes of these cells are so different from CG7 cells which are defective in receptor recycling. Nevertheless, this model integrates the data presented in this report with previous observations and should provide a focus for future studies of the import process.

Peroxisomal import of an intact receptor-ligand complex, either as a normal step in import or as a consequence of a mutant translocation factor, would require formation of a prohibitively large protein translocation pore. However, peroxisomal import of oligomeric protein complexes (Glover et al., 1994; McNew and Goodman, 1994) and large, nondeformable structures such as PTS1 peptide-coated gold particles (Walton et al., 1995) have been observed. A key question is whether such structures are accommodated through the normal translocation pore or whether they represent an artifactual form of import. Identification of the gene defective in CG7 cells, the nature of the inactivating mutations in these cells, and an analysis of the CG7 gene product should shed light on the mechanism of protein translocation through the peroxisome membrane.

In addition to the altered subcellular distribution of Pex5p in cell lines from PBD complementation groups 3, 10, and 7, we observed that the steady-state abundance of Pex5p was severely reduced in CGs 1, 4, and 8. PEX5 is not mutated in these cell lines and overexpression of PEX5 cannot rescue their peroxisomal protein import defects (Dodt et al., 1995). The requirement for multiple gene products for Pex5p stability resembles the instability

of the α, β, and γ sarcoglycan proteins in muscular dystrophy patients who carry mutations in the dystrophin gene or any one of the α, β, or γ sarcoglycan genes (Bonnemann et al., 1995; Ervasti et al., 1990; Lim et al., 1995; Noguchi et al., 1995). These proteins are all components of the dystrophin-glycoprotein complex, a high molecular mass oligomeric complex. Based on this precedent, it may be that Pex5p physically interacts with the products of the CG1, CG4, and CG8 genes. Of these, only the CG4 gene (PEX6) has been identified. PEX6 encodes a AAA ATPase that is required for stability of the PTS1 receptor in yeast as well as in human cells (Yahraus et al., 1996; Reuber, B., and S.J. Gould, manuscript in preparation). Characterization of the CG1, 4, and 8 genes and gene products in cells from both normal and affected individuals will provide us with a better understanding of peroxisomal protein import.

CG11 cells display a PTS2-specific import defect, carry inactivating mutations in the PTS2 receptor gene, PEX7 (Braverman, N., S.J. Gould, and D. Valle, manuscript in preparation), and import PTS1 proteins normally (Motley et al., 1994; Slawecki et al., 1995). We found no difference in the distribution or abundance of Pex5p in CG11 cells as compared to normal fibroblasts. The fact that altered distribution and/or abundance of Pex5p is restricted to those PBD cell lines with a defect in import of PTS1 proteins, and is not observed in cells with a specific defect in PTS2 protein import provides further evidence that the CG1, PEX12 (CG3), PEX6 (CG4), CG7, CG8, and PEX2 (CG10) gene products all play direct roles in peroxisomal matrix protein import.

In its present state, our model of peroxisomal protein import resembles protein import into mitochondria (Ryan and Jensen, 1995), the nucleus (Powers and Forbes, 1994), and the endoplasmic reticulum (Walter and Lingappa, 1986). All four import pathways use a predominantly cytoplasmic targeting signal receptor that can cycle between the cytoplasm and organelle. The ubiquitous use of cytoplasmic receptors for directing newly synthesized organellar proteins to their proper destination implies that diffusion alone cannot mediate the transport of newly synthesized proteins from the cytoplasm to their appropriate target organelle. However, if diffusion cannot mediate transport of proteins to target organelles, there is no reason to presume that diffusion alone could mediate transport of receptor-ligand complexes to the peroxisome. Therefore, it may be that multiple factors are involved in directing the PTS1 receptor-ligand complex from the cytoplasm to the peroxisome. It will be particularly interesting to determine whether the same gene products required for Pex5p stability are also involved in assembling this complex, directing it to the peroxisome or dissembling it once it docks to the peroxisome surface.

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