

Differential Protein Import Deficiencies in Human Peroxisome Assembly Disorders

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Abstract. Two peroxisome targeting signals (PTSs) for matrix proteins have been well defined to date. PTS1 comprises a COOH-terminal tripeptide, SKL, and has been found in several matrix proteins, whereas PTS2 has been found only in peroxisomal thiolase and is contained within an NH₂-terminal cleavable presequence. We have investigated the functional integrity of the import routes for PTS1 and PTS2 in fibroblasts from patients suffering from peroxisome assembly disorders. Three of the five complementation groups tested showed a general loss of PTS1 and PTS2 import. Two complementation groups showed a differential loss of peroxisomal protein import: group I cells were able to import a PTS1- but not a PTS2-containing reporter protein into their peroxisomes, and group IV cells were able to import the PTS2 but not

the PTS1 reporter into aberrant, peroxisomal ghostlike structures. The observation that the PTS2 import pathway is intact only in group IV cells is supported by the protection of endogenous thiolase from protease degradation in group IV cells and its sensitivity in the remaining complementation groups, including the partialized disorder of group I. The functionality of the PTS2 import pathway and colocalization of endogenous thiolase with the peroxisomal membranes in group IV cells was substantiated further using immunofluorescence, subcellular fractionation, and immunoelectron microscopy. The phenotypes of group I and IV cells provide the first evidence for differential import deficiencies in higher eukaryotes. These phenotypes are analogous to those found in *Saccharomyces cerevisiae* peroxisome assembly mutants.

PEROXISOMES are ubiquitous subcellular organelles bounded by a single membrane. Although their protein content varies depending on the organism and cell type (Tolbert, 1981), a feature common to all peroxisomes is the presence in the matrix of hydrogen peroxide-producing oxidases and other enzymes required for β -oxidation, and catalase. A variety of other biochemical functions has been assigned to peroxisomes (Lazarow, 1987; Van den Bosch et al., 1992; Mannaerts and Van Veldhoven, 1993), and their importance for cellular metabolism has been underlined by the occurrence of severe disorders in man caused by deficiencies in peroxisomal functions (Kelley et al., 1986; Schutgens et al., 1986; Wanders et al., 1988; Lazarow and Moser, 1989; Zellweger, 1989). These diseases fall into three categories: (a) the generalized disorders of peroxisome assembly, in which all peroxisomal functions investigated are deficient; (b) the partialized disorders of peroxisome assembly, in which the activity of a limited subset of peroxisomal enzymes is deficient; and (c) disorders caused by a deficiency of a single peroxisomal enzyme.

Complementation studies based on the recovery of peroxisomal function after fusion of certain combinations of patients' fibroblasts indicate that the peroxisome assembly disorders are genetically heterogeneous (Brul et al., 1988; Roscher et al., 1989; Poll-The et al., 1990; Yajima et al., 1992). The complementation groups established by three research groups have recently been integrated (Shimozawa et al., 1993), and we have investigated protein import into peroxisomes in four of these complementation groups. The fibroblasts belonging to these complementation groups, which we refer to using the Amsterdam nomenclature, are derived from patients suffering from the generalized impairments of peroxisome assembly. Morphologically distinguishable peroxisomes, visualized as particle-bound catalase, are virtually absent (Goldfischer et al., 1973). Immunoblots have shown that the levels of the β -oxidation enzymes are strongly reduced (Tager et al., 1985; Suzuki et al., 1986; Suzuki et al., 1988), and these proteins are thought to be degraded in the absence of import into peroxisomes. At least some peroxisomal membrane proteins, however, are present in atypical structures that have been termed ghosts (Santos et al., 1988a, 1988b; Wiemer et al., 1989). In electron micrographs, these structures are two to four times larger than peroxisomes from control cells, and they appear to lack content (Santos et al., 1988a). Subcellular fractionation experiments have shown that peroxisomal

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ghosts fractionate at a lower density than peroxisomes from control cells (Santos et al., 1988a; Gartner et al., 1991; Van Roermund et al., 1991).

In addition to the generalized disorders of peroxisome assembly, in our analysis, we have also included cells derived from patients suffering from a partialized disorder of peroxisome assembly: rhizomelic chondrodysplasia punctata (RCDP)¹ (Heymans et al., 1985 and 1986; Hoeffler et al., 1988; Poulos et al., 1988), which comprises our complementation group I (Brul et al., 1988; Heikoop et al., 1992c). Classical RCDP is characterized biochemically by the following features: first, there is a deficiency of the first two enzymes of plasmalogen biosynthesis; second, there is an inability to catabolize phytanic acid, and finally, there is a failure to convert the precursor form of thiolase to the mature form of the enzyme. The morphology of peroxisomes, however, appears to be normal in RCDP fibroblasts (Heikoop et al., 1991), and catalase is present in the matrix. Subcellular fractionation has shown that the peroxisomes from RCDP fibroblasts fractionate at the same density as peroxisomes from control cells, while precursor thiolase is present in lower density fractions in RCDP cells (Balfe et al., 1990; Singh et al., 1991; Heikoop et al., 1992a).

The aim of our research was to further analyze the mutant phenotypes in each of the complementation groups by investigating the functional integrity of the import routes for the two well-defined peroxisome targeting signals (PTS) for matrix proteins: the PTS1 is comprised of a COOH-terminal tripeptide, SKL, and derivatives thereof. The PTS1 was first found at the COOH terminus of the firefly enzyme luciferase (Gould et al., 1987), and the PTS1 has since been found on a number of peroxisomal matrix proteins (Gould et al., 1988). The PTS2 was first identified on rat 3-ketoacyl-CoA thiolase (Swinkels et al., 1991; Osumi et al., 1991), and is located within an NH₂-terminal sequence that in mammals is cleavable. That proteins containing either PTS1 or PTS2 enter the peroxisome by different import routes is reflected by the phenotypes of some of the yeast peroxisome assembly mutants: *Saccharomyces cerevisiae pas7* can import SKL proteins but not thiolase into its peroxisomes (Van der Leij et al., 1992; Erdmann and Kunau, 1993), and *S. cerevisiae pas10* (Van der Leij et al., 1992, 1993) and *Pichia pastoris pas8* (McCollum et al., 1993) can import thiolase but not SKL proteins. In other complementation groups, neither thiolase nor SKL proteins are imported (Van der Leij et al., 1992; Zhang et al., 1993). How the peroxisomal membrane proteins are targeted to the organelle is still unknown, and additional PTS motifs for this class of proteins remain to be identified.

We have investigated import via the PTS1 in human fibroblasts using luciferase as a reporter protein. A prethiolase-chloramphenicol acetyltransferase (CAT) fusion protein was used as a reporter for import via the PTS2 route. The reporter proteins were produced from expression constructs introduced into the nucleus of cells by microinjection. Our investigation into the PTS1 and PTS2 import routes

shows that phenotypes analogous to those of the yeast mutants can be found among the human peroxisome assembly disorders. The observation that either one of the import routes can be deficient provides the first evidence that the two import routes for matrix proteins can be impaired independently of each other in the human peroxisome assembly disorders.

Materials and Methods

Cell Cultures

Cultured primary skin fibroblasts used in these studies were from two control subjects (cell lines 85AD5035F and HENG90AD) and from patients suffering from the clinical and biochemical features characteristic of the following peroxisomal disorders: (a) RCDP, complementation group I (cell lines MCHE 85AD and GJOLD90AD); (b) Zellweger syndrome, complementation group II (cell lines W78/515 and BOV 84AD), group III (GOM 85AD and GM 4340), and group V (GRO 87AD); (c) hyperpipecolic acidemia, group II (cell line GM 3605); (d), the neonatal form of adrenoleukodystrophy, group IV (cell line AAL 85AD). Cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F10 (Gibco Laboratories, Glasgow, United Kingdom) supplemented with 10% fetal calf serum (Gibco Laboratories) under 5% CO₂. Cells were split 1:4 as soon as they reached confluence, as allowing the cells to remain confluent has been shown to increase the amount of immunoreactive thiolase in the cells (Wiemer et al., 1991). Cells were not used above passage 30, as an increased frequency of punctate fluorescence has been observed in aging cells (unpublished observations).

Microinjection

24 h before microinjection, the cells were plated onto microinjection grids. The needles were made using a micropipette puller (PB-7; Narishage Co., Tokyo), and DNA was injected in reverse PBS (4 mM Na₂HPO₄/1 mM KH₂PO₄/140 mM KCl, pH 7.3). DNA was injected at a concentration of ~0.1 µg/ml, which is equivalent to ~50 mol DNA per injection. 18 h after microinjection, the cells were processed for immunofluorescence microscopy. Approximately 200 cells were injected per cell line per experiment, ~20% of which survived injection and gave rise to detectable expression.

Subcellular Fractionation

Seven to nine 150-cm² flasks of cells were used per gradient. The cells were harvested by gentle trypsinization and collected by low-speed centrifugation. The cells were then washed twice in PBS and once in ice-cold homogenization buffer (0.25 mM sucrose/0.1 mM EDTA/1 mM Tris-HCl, pH 7.8), and postnuclear supernatants were prepared as previously described (Van Roermund et al., 1991). Subsequently, equilibrium density centrifugation was performed as described (Van Roermund et al., 1991) by layering 4 ml of the postnuclear supernatant (containing ~25 mg of protein) on top of a preformed 33-ml gradient of Nycodenz (10–35%). All Nycodenz solutions were prepared in homogenization buffer. Centrifugation was carried out in an MSE-Europe 24M centrifuge (M.S.E. Scientific Instruments, Crawley, United Kingdom) equipped with a vertical rotor for 90 min at 19,000 rpm at 4°C. Fractions were collected from the bottom of the gradient and analyzed for the activity of the marker enzymes glutamate dehydrogenase (mitochondria), catalase (peroxisomes), and β-hexosaminidase (lysosomes). Activities are expressed as percentages of total activity across the gradient.

Proteinase K Protection

The proteinase K protection experiments were performed on fibroblasts permeabilized by 125 µg/ml digitonin in permeabilization buffer. After harvesting the cells by trypsinization, the fibroblasts were washed twice in cold PBS and resuspended to a protein concentration of ~1 mg/ml in isotonic sucrose buffer (250 mM sucrose, 20 mM MOPS, pH 7.4), containing 125 µg/ml digitonin (permeabilization buffer). The fibroblasts were then incubated for 5 min at 37°C in the digitonin permeabilization buffer, as previously described (Heikoop et al., 1990). This concentration of digitonin causes rupture of the plasma membrane (and perhaps also some intracellu-

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; PAF, peroxisome-assembly factor; PMP, peroxisome membrane protein; PTS, peroxisome targeting signal; RCDP, rhizomelic form of chondrodysplasia punctata; TRITC, Texas red isothiocyanate.

lar membranes) (Wanders et al., 1984), whereas the peroxisomal membrane remains intact (Wanders et al., 1984; Aikawa et al., 1991). This is indicated by the fact that the cytosolic marker lactate dehydrogenase was present in the soluble fraction, whereas catalase activity was present in the sedimentable fraction (10,000 *g*, 5 min) of both control and RCDP cells. After permeabilization of the plasma membrane, the protease protection assay was carried out on ice using a proteinase K concentration of 40 $\mu\text{g/ml}$ in the presence or absence of 0.2% Triton X-100. Samples were removed after 0.5, 1, and 1.5 h, and proteinase K action was stopped by addition of PMSF to 10 mM. Protection of thiolase was assessed by immunoblot analysis.

Immunoblot Analysis

Samples were subjected to electrophoresis on 10% polyacrylamide gels according to the method of Laemmli. Immunoblot analyses were performed as described previously with the following modifications: Nonspecific binding sites were blocked overnight with 10% fetal calf serum in TBS (10 mM Tris pH 8/150 mM NaCl) containing 0.2% Tween 20. The primary (polyclonal antithiolase) (Heikoop et al., 1990) and secondary (horse radish peroxidase coupled to goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA) antibody incubations were performed in TBS containing 4% fetal calf serum and 0.1% Tween 20. After each incubation, the blots were washed extensively in TBS containing 0.1% Tween 20. Antigen-antibody complexes were visualized using the electrochemiluminescence kit from Amersham International (Den Bosch, The Netherlands).

Immunofluorescence Microscopy

For labeling of the peroxisomal compartment, cells were washed in PBS and fixed in freshly prepared 2% (wt/vol) *p*-formaldehyde/0.1% Triton X-100/PBS (pH 7.5) for 20 min at room temperature. For differential permeabilization, the cells were fixed in 2% paraformaldehyde in PBS for 20 min and permeabilized with 25 $\mu\text{g/ml}$ digitonin in PBS (which permeabilizes the plasma membrane only) or 25 $\mu\text{g/ml}$ digitonin in PBS containing 0.1% Triton X-100 (to permeabilize both the plasma membrane and the peroxisomal membrane). 25 $\mu\text{g/ml}$ digitonin is sufficient to permeabilize the plasma membrane of adhering cells because of the relatively low protein concentration (125 $\mu\text{g/ml}$ was used to permeabilize cells in suspension at a protein concentration of 1 mg/ml [see section on "Proteinase K Protection"]). For double labeling of peroxisomes and lysosomes, the cells were fixed by a 10-min incubation at room temperature in 4 ml 3% paraformaldehyde in PBS, pH 7.5, followed by a 5-min incubation after addition to the 4 ml paraformaldehyde of 2 ml cold (-20%) methanol, followed by a final 5-min incubation after a further addition of 2 ml cold methanol. The fixative was then aspirated, and the cells were washed three times in PBS containing 0.05% Triton X-100.

Free aldehyde groups were blocked by incubating for 10 min in 0.1 M NH_4Cl in PBS. The cells were then processed for single- or double-label indirect immunofluorescence using the following antibodies: mouse monoclonal antithiolase (3G4) (Heikoop et al., 1991), polyclonal anti-69-kD peroxisomal membrane protein (PMP) (Koster et al., 1986) (a kind gift from Dr. W. Just, Institut für Biochemie, Heidelberg, Germany), polyclonal (guinea pig) antiluciferase (Keller et al., 1987), and polyclonal (rabbit) anti-chloramphenicol acetyltransferase (Sanvertech, Boechout, Belgium). For double labeling of luciferase and 69-kD PMP, 69-kD PMP was visualized with FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA), and luciferase was visualized using biotinylated goat anti-guinea pig IgG (Sigma Immunochemicals, Bornem, Belgium) followed by streptavidin-labeled Texas red isothiocyanate (TRITC) (Amersham International). For double labeling of either 69-kD PMP or prethiolase-CAT with monoclonal antithiolase antibodies, the secondary antibodies used were FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and biotinylated sheep anti-mouse Ig (Amersham International), followed by streptavidin-labeled TRITC (Amersham International). For double labeling of peroxisomal thiolase and lysosomal glucocerebrosidase, peroxisomal thiolase was visualized with biotinylated sheep anti-mouse Ig (Amersham International) followed by streptavidin-labeled TRITC (Amersham International), and lysosomes were visualized using rabbit antiglucocerebrosidase (kind gift from Dr. H. Aerts, E. C. Slater Institute, Amsterdam) followed by FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). For single labeling, biotinylated secondary antibodies were used followed by streptavidin-labeled FITC (Life, Breda, The Netherlands).

The incubations were all performed at room temperature for 45 min in PBS containing 10 mg/ml bovine serum albumin (to reduce nonspecific

binding). After each incubation, the cells were washed extensively in PBS. The injection grids were mounted on coverslips in carbonate-buffered glycerol containing *p*-phenylenediamine. The fluorescent staining pattern was viewed in a fluorescence microscope (model IMT2; Olympus Corp., Lake Success, NY), using an IMT2DMB filter for excitation of the FITC signal, and an IMT2-DMG for excitation of the TRITC signal. FITC fluorescence was visualized in combination with the G520 barrier filter and TRITC fluorescence in combination with the B610 barrier filter. Fluorescent images were recorded on AGFA 400 ASA slide film or Kodak TMAX 400 ASA B/W film.

Electron Microscopy

Fixation of fibroblasts and preparation of ultrathin cryosections were performed exactly as described by Wiemer et al. (1989). Immunolabeling was performed as described previously with the following modifications. For double labeling with anti-69 kD PMP (rabbit polyclonal) and antithiolase (mouse monoclonal), the sections were first incubated with antithiolase followed by rabbit anti-mouse IgG, followed by protein A-conjugated, 10-nm colloidal gold. Residual binding sites were blocked with protein A. Secondly, the sections were incubated with anti-69 kD PMP followed by protein A conjugated with 5 nm of colloidal gold. After each incubation the grids were washed with PBS. The tissue sections mounted on the grids were poststained and embedded in a 0.3% uranyl acetate/1.5% methylcellulose mixture and dried in air. Grids were examined under a transmission electron microscope (model 420; Philips Electronic Instruments, Inc., Mahwah, NJ).

Construction of Vectors

The construction of pCDNALuc was as follows: the luciferase ORF was cut out of pDDR (Riggs and Chrispeels, 1987) with *Sma*I and *Hind*III, and the ends of this fragment were made blunt with the Klenow fragment of DNA polymerase I. *Bst*XI adaptors were ligated to this fragment, which was then cloned into the *Bst*XI site of the expression vector pCDNA. The expression plasmid encoding the first 15 amino acids of rat thiolase fused onto bacterial chloramphenicol acetyltransferase (pRSV-FLT3-CAT) was a kind gift from S. Subramani (University of California, San Diego, CA) and is described by Swinkels et al. (1991).

Results

Our strategy to investigate peroxisomal protein import in the peroxisome assembly disorders involved microinjection into the nucleus of expression constructs encoding reporter proteins for the PTS1 or PTS2 import routes. We chose to microinject DNA rather than RNA or protein because the prolonged expression period after microinjection of DNA allows the cells time to recover from the trauma of microinjection before detection of the antigen by immunofluorescence. Expression of luciferase could still be detected 48 h after microinjection of the DNA. Microinjection trauma is minimized because only ~50 molecules of DNA need to be

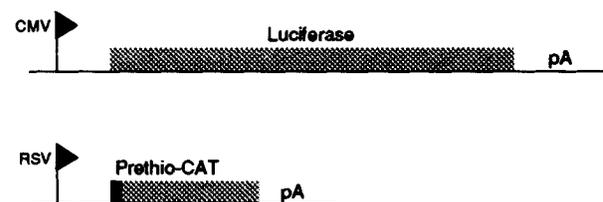


Figure 1. Constructs used to determine import via the PTS1 (luciferase) or PTS2 (prethiolase-CAT) import routes. Stippled boxes, reading frames of the reporter proteins; thin lines, plasmid sequences; \blacktriangleright , the promoter (CMV, cytomegalovirus; RSV, Rous sarcoma virus); pA, the polyadenylation sequence. For details of construction, see Materials and Methods.

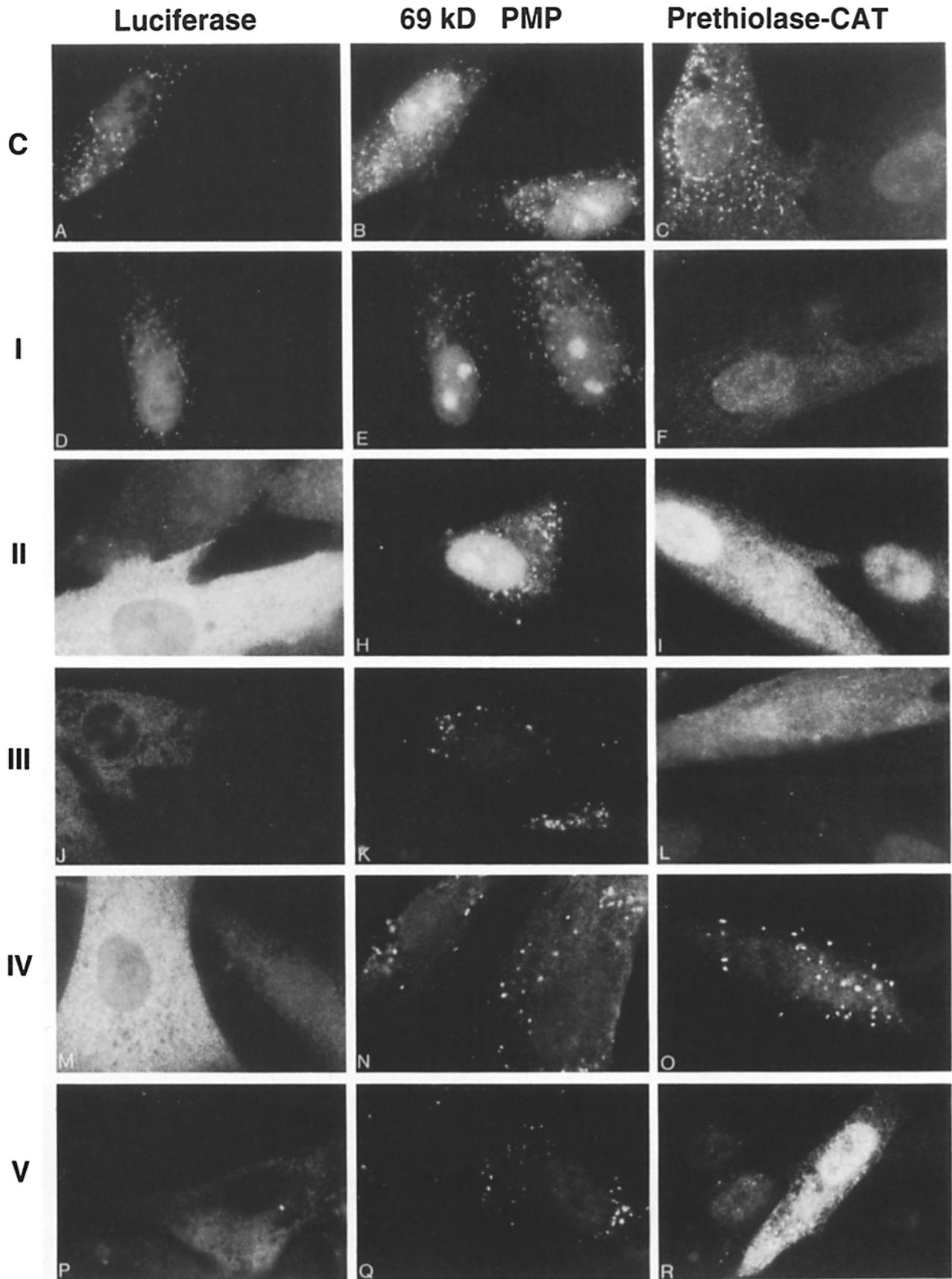


Table 1. Targeting of Luciferase and Prethiolase-CAT in Complementation Groups I-V

Complementation group according to nomenclature of:			Targeting of luciferase	Targeting of prethiolase-CAT
Amsterdam	Baltimore	Gifu		
I (RCDP)			+*	-
II	1	E	-	-
III	4	C	-	-
IV	2	-	-	±†
V	-	F	-	±‡

Our complementation groups have been integrated with those of Baltimore (Roscher et al., 1989) and Gifu (Yajima et al., 1992) by Shimozawa et al. (1993).

* Import into peroxisomes that appear morphologically normal and contain catalase (Heikoop et al., 1991).

† Import into peroxisomal ghosts (Santos et al., 1988a and 1988b; Wiemer et al., 1989). Targeting of prethiolase-CAT to peroxisomal ghosts in group V cells is very weak and can only be detected in 25-50% of the cells expressing the reporter protein.

injected per cell to assess import. The high sensitivity of the system is a reflection of the amplification by transcription and translation steps, and of the strength in human fibroblasts of the promoters used (cytomegalovirus and Rous sarcoma virus) (Fig. 1).

PTS1 comprises a COOH-terminal tripeptide, SKL, and firefly luciferase was chosen as the reporter protein for this import route. A prethiolase-CAT fusion protein was used as the reporter protein for import via the PTS2 route. Targeting was assessed by processing the cells for immunofluorescence 18 h after microinjection of the expression constructs encoding the reporter proteins. In control cells, expression of both the PTS1 and PTS2 reporter proteins gave rise to a punctate pattern of labeling (Fig. 2, A and C), which was taken to be indicative of targeting: targeting of the reporter proteins to a peroxisomal compartment was assessed by double-label immunofluorescence using antibodies directed against the 69-kD PMP, a well-established marker of the peroxisomal membrane (Fig. 2 B).

Targeting via the PTS1 and PTS2 Routes in Groups I-V

Luciferase expressed in complementation group I cells gave rise to a pattern of labeling indistinguishable from that in control cells, and the peroxisomal nature of this compartment was confirmed by colocalization with the 69-kD peroxisomal membrane protein (Fig. 2, D and E). Complementation groups II-V all contain peroxisomal ghosts (Fig. 2, H, K, N, and Q, respectively), indicating that targeting of the 69-kD peroxisomal membrane protein is still functional in the complementation groups used in this study. However, luciferase expressed in cells from groups II-V gave rise to a

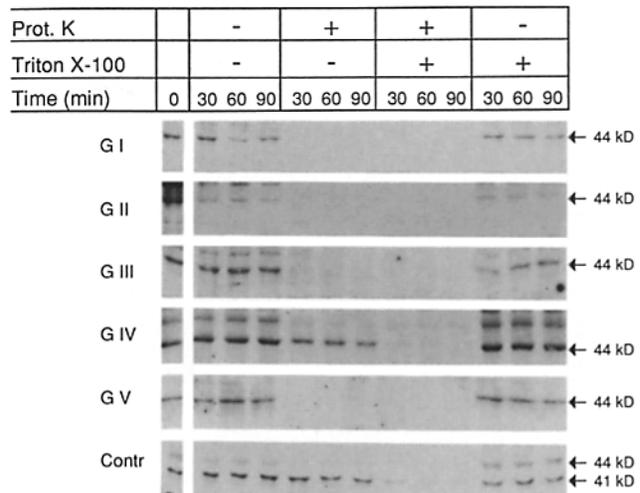
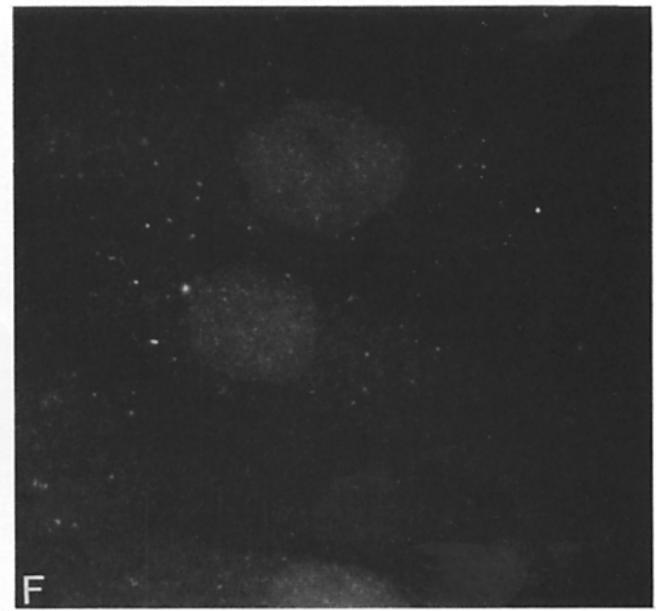
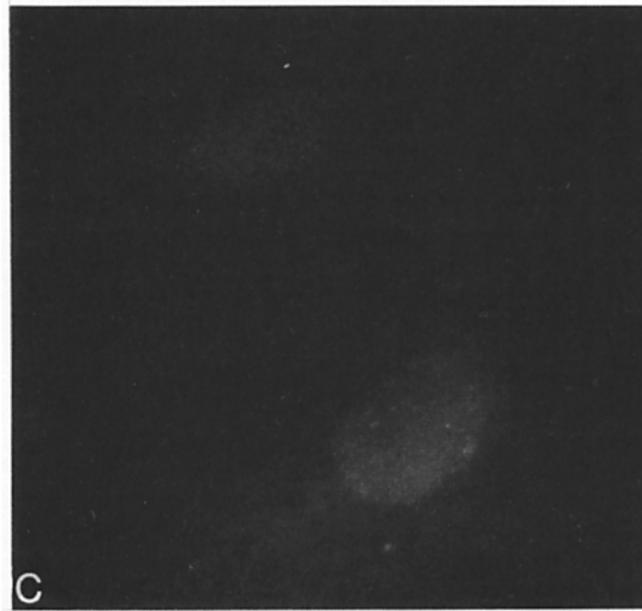
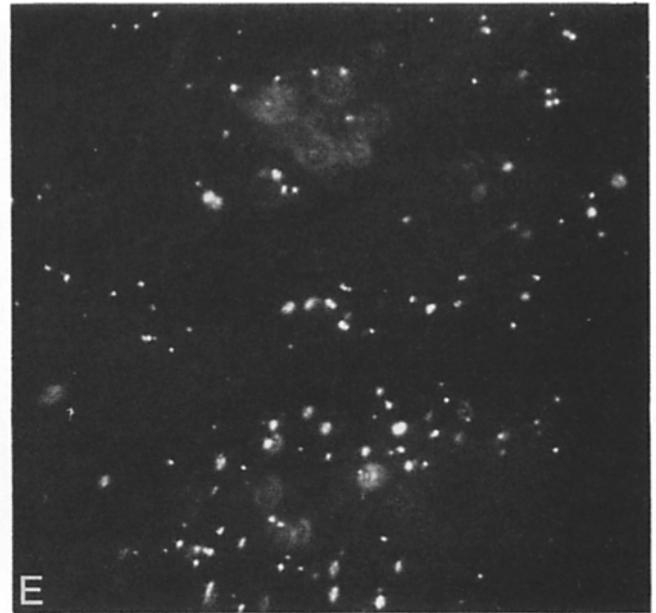
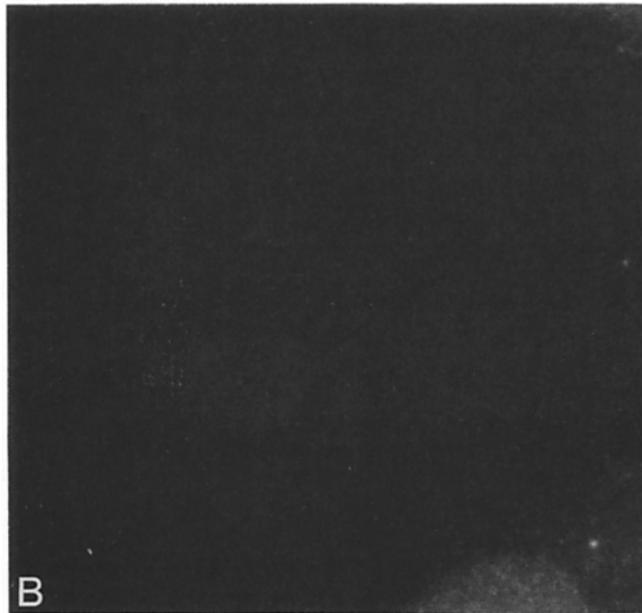
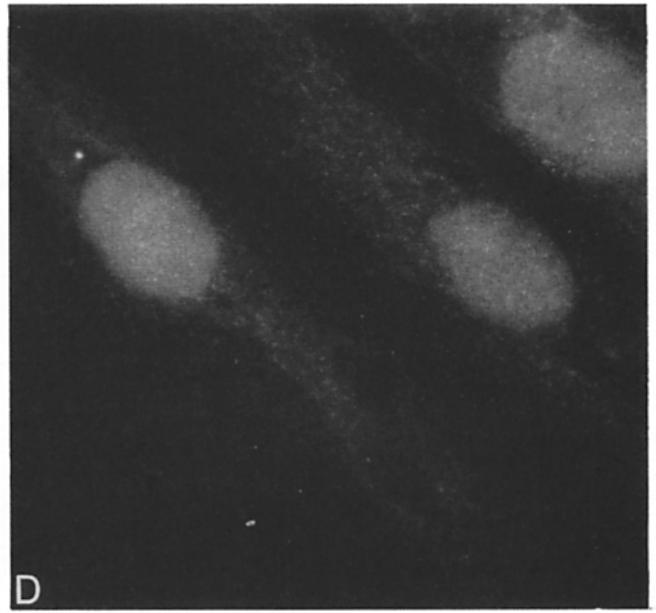
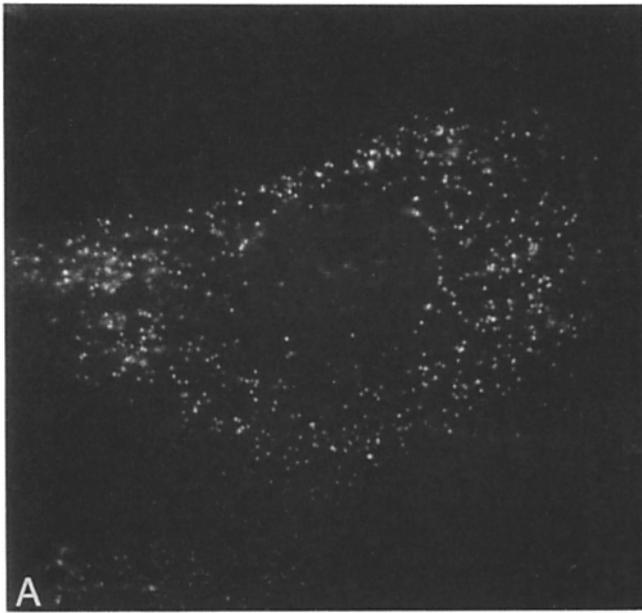


Figure 3. Precursor thiolase is protected from degradation by proteinase K in group IV cells only. Fibroblasts from a control subject and from each of the complementation groups were permeabilized with 125 μ g/ml digitonin and incubated at 4°C for the times indicated in the presence or absence of 40 μ g/ml proteinase K and Triton X-100, which was added to disrupt intracellular membranes. 0.2% Triton was sufficient to observe digestion of precursor thiolase in group IV cells, whereas 0.5% Triton was required before digestion of mature thiolase was observed in control cells. The arrows indicate the positions of precursor thiolase (44 kD) and mature thiolase (41 kD).

pattern of labeling characteristic for a protein with a cytoplasmic location (Fig. 2, G, J, M, and P), and no association with the peroxisomal membranes could be detected. Identical patterns of staining were seen after expression of CAT-SKL (not shown), a second reporter protein for the SKL import route.

Expression of prethiolase-CAT in cells from complementation groups I-III gave rise to a diffuse pattern of fluorescence (Fig. 2, F, I, and L); no association with the peroxisomal membranes could be detected, not even in complementation group I fibroblasts, which contain morphologically normal, catalase- and luciferase-positive peroxisomes. Prethiolase-CAT expressed in complementation group V fibroblasts gave rise to a weak particulate pattern of labeling in only some (25-50%) of the cells expressing the reporter protein (Fig. 2 R), the expression pattern being diffuse in the remaining cells. In group IV cells, however, the PTS2 reporter protein gave rise to a strong particulate pattern of labeling in virtually all the cells expressing the reporter protein (Fig. 2 O): the structures labeled resemble peroxisomal ghosts (Fig. 2 N). These results are summarized in Table I.

Figure 2. Punctate fluorescence as an indication for import of the reporter proteins into peroxisomes. The subcellular localization of luciferase (left panels), 69-kD PMP (center panels), and prethiolase-CAT (right panels) is shown in fibroblasts from a control subject (C) and from complementation groups I-V (I-V). Luciferase (PTS1 reporter) and prethiolase-CAT (PTS2 reporter) were produced in the cells from microinjected expression constructs. Double labeling in control (A, B) and group I (D, E) cells shows that luciferase colocalizes with the 69-kD PMP, whereas the pattern of immunofluorescence in groups II-V expressing luciferase is diffuse (G, J, M, and P). Expression of prethiolase-CAT gives rise to a pattern of fluorescence that in groups I-III is diffuse (F, I, and L), in group IV is particulate (O), and in group V is sometimes weakly particulate (R) and sometimes diffuse.



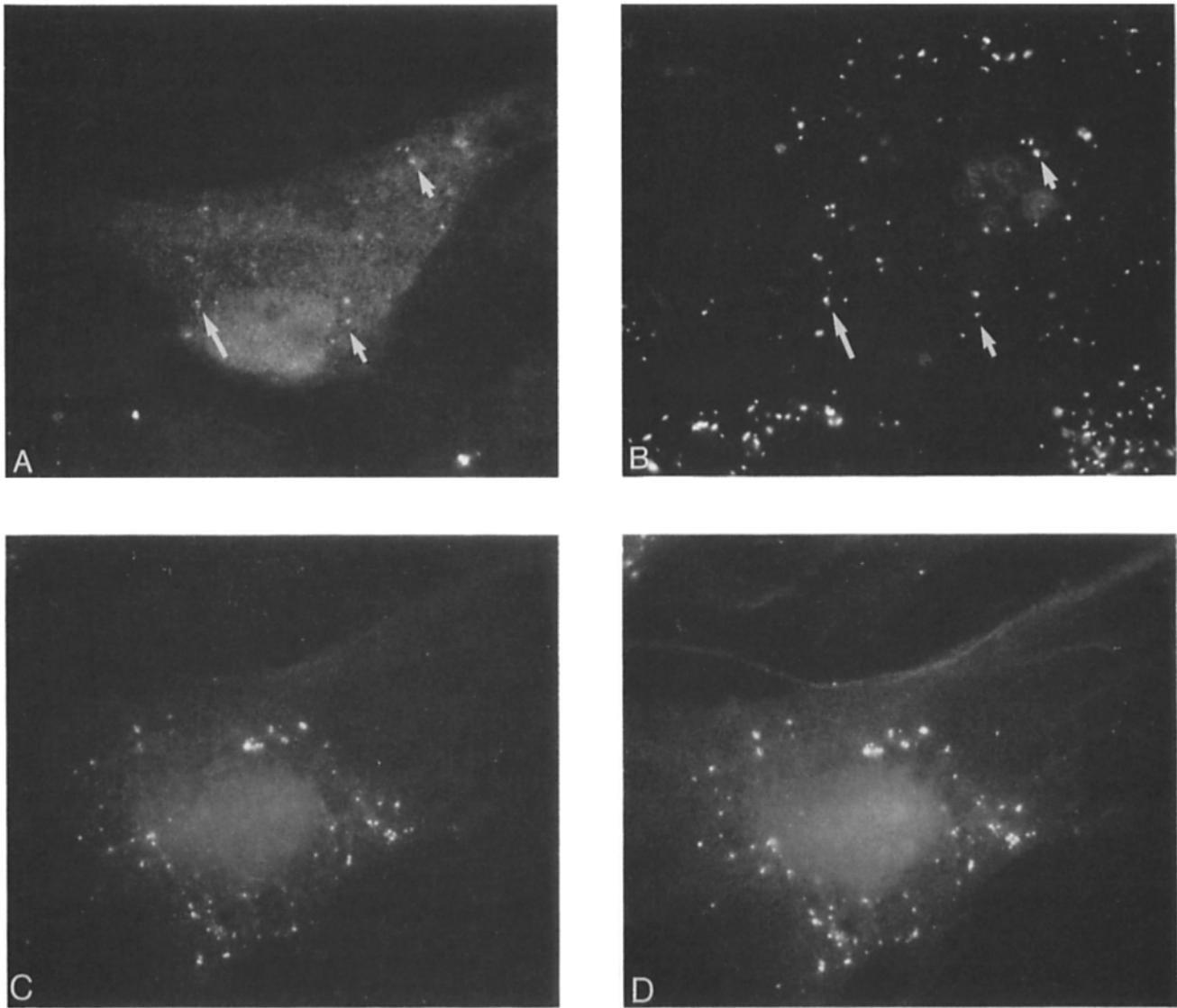


Figure 5. Colocalization of prethiolase-CAT, 69-kD PMP and endogenous thiolase using double-label immunofluorescence. Prethiolase-CAT (A) colocalizes with endogenous thiolase (B) in group IV cells expressing the PTS2 reporter protein, and endogenous thiolase (C) colocalizes with 69-kD PMP in group IV cells (D).

Protease Protection of Endogenous Thiolase in Groups I-V

If import via the PTS2 pathway is intact, thiolase will be protected from protease digestion because of its presence within the peroxisomal membrane. Protease protection experiments were performed on digitonin-permeabilized cells to investigate the stability of endogenous thiolase in the five complementation groups. Digitonin disrupts membranes by complexing to cholesterol: we have used 125 $\mu\text{g/ml}$ digitonin, and under these conditions, the plasma membrane (and other cholesterol-rich membranes) are permeabilized, but

the peroxisomal membrane, which contains relatively little cholesterol, remains intact (Wanders et al., 1984; Aikawa et al., 1991). As shown in Fig. 3, fibroblasts from complementation groups I-V contain only the precursor form of thiolase (44 kD). In each of the complementation groups except group IV, precursor thiolase was degraded in the absence of Triton X-100; in complementation group IV, however, precursor thiolase remained protected from protease digestion at each of the time points investigated, which suggests that it is present within a membrane.

Because the immunofluorescence and protease protection experiments suggest that targeting and import via the PTS2

Figure 4. Detection of endogenous thiolase using a monoclonal antibody. (A) Control fibroblasts; (B-F) fibroblasts from groups I-V, respectively. A typical peroxisomal labeling pattern is seen in control cells (A), a very weak, diffuse pattern of labeling is found in groups I-III (B-D), a strong particulate pattern of labeling in group IV (E), and a weak pattern of labeling in group V cells (F), which in some cells is particulate and in some cells is diffuse.

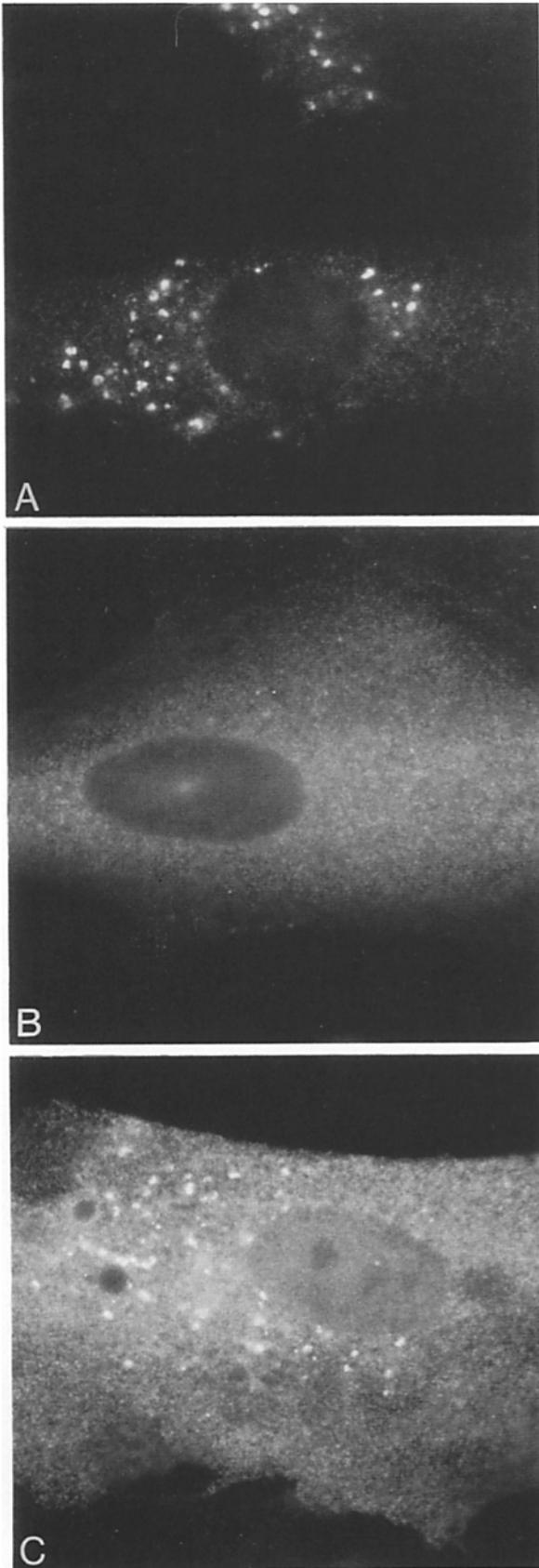


Figure 6. Differential permeabilization shows that the PTS2 reporter protein is present inside the peroxisomal membranes in group IV cells. Differential permeabilization was performed using

pathway are intact in group IV cells, additional experiments were performed to further substantiate these observations.

Confirmation of Thiolase Targeting to Peroxisomes in Group IV Cells

The observations made using the PTS2 reporter protein were supported when endogenous thiolase in the cells was visualized using a monoclonal antibody directed against peroxisomal thiolase. When processed for antithiolase immunofluorescence, cells from complementation groups I–III showed a very weak, diffuse pattern of labeling (Fig. 4, B, C, and D, respectively) (Heikoop et al., 1992c). In complementation group V cells, a weak pattern of labeling was seen, which in some cells was particulate and in some cells was diffuse (Fig. 4 F). In complementation group IV cells, however, a strong particulate pattern of labeling was seen, and the structures labeled resembled peroxisomal ghosts (Fig. 4 E). Double-labeling immunofluorescence showed that prethiolase-CAT expressed after microinjection (Fig. 5 A) colocalizes with endogenous thiolase in group IV cells (Fig. 5 B).

To determine whether endogenous thiolase colocalizes with the peroxisomal membranes in group IV cells, double-labeling immunofluorescence using the antithiolase monoclonal and the anti-69 kD PMP polyclonal antibody was performed. Fig. 5 shows that the thiolase immunoreactivity in group IV cells (C) indeed colocalizes with the peroxisomal membranes (D).

Thiolase is Imported into Peroxisomal Membranes in Group IV Cells

Differential permeabilization with digitonin (25 µg/ml) followed by immunofluorescence was used to determine whether prethiolase-CAT is present on the inside or the outside of the peroxisomal membrane in group IV cells expressing the PTS2 reporter protein. When the plasma membrane was permeabilized using low concentrations of digitonin, epitopes exposed only to the cytoplasm were accessible to labeling by an antibody. Under these conditions, antibodies directed against the 69-kD PMP labeled the peroxisomal membranes (Fig. 6 A), whereas a diffuse pattern of labeling was observed using anti-CAT antibodies in cells expressing the PTS2 reporter protein (Fig. 6 B). When Triton X-100 was included in the permeabilization, both the plasma membrane and the peroxisomal membrane were permeabilized. Under these conditions, particulate prethiolase-CAT could be detected (Fig. 6 C). The same results were obtained using an antithiolase monoclonal antibody to detect endogenous thiolase (not shown); thiolase labeling could be detected

25 µg/ml digitonin, which permeabilizes the plasma membrane only, or digitonin plus Triton X-100, which permeabilizes both the plasma membrane and the peroxisomal membrane. (A) Staining for 69-kD PMP labels the peroxisomal membranes in group IV cells permeabilized with 25 µg/ml digitonin. (B) When the PTS2 reporter protein is labeled in group IV cells permeabilized with 25 µg/ml digitonin, a diffuse pattern of labeling is seen. (C) When Triton and digitonin are used to permeabilize group IV cells expressing the PTS2 reporter protein, a particulate pattern of labeling of prethiolase-CAT can be detected.

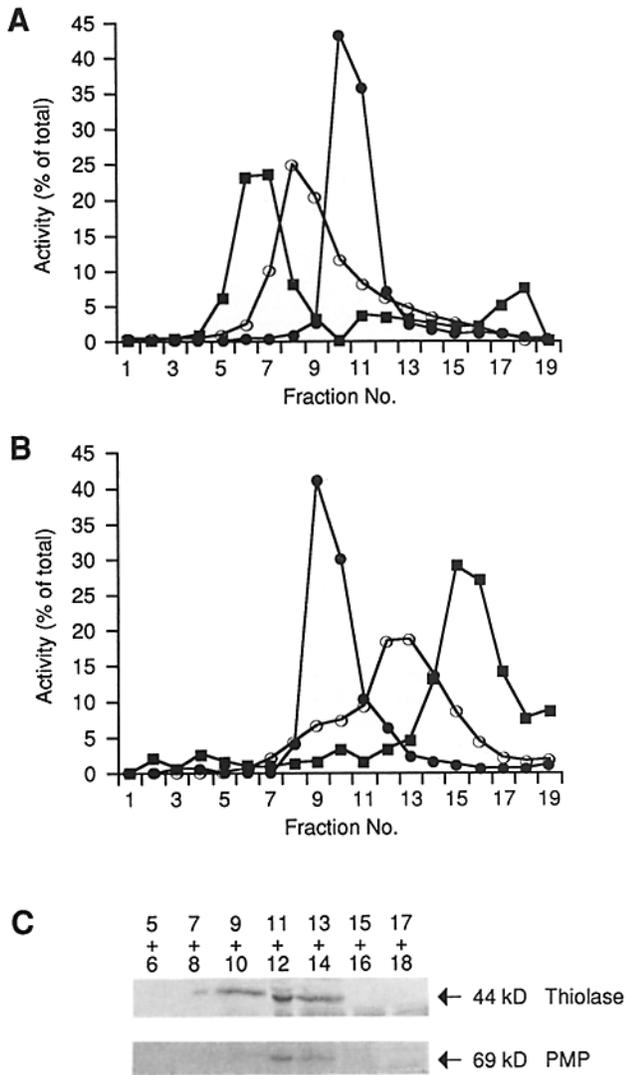


Figure 7. Subcellular fractionation on Nycodenz equilibrium gradients shows that the peroxisomal "ghosts" in group IV cells equilibrate in a low density fraction. Fractionation of (A) control and (B) group IV fibroblasts. ■, catalase; ●, glutamate dehydrogenase (mitochondria); ○, β -hexosaminidase (lysosomes). Catalase (and other peroxisomal enzymes [not shown]) peak in fractions 6 and 7 in control cells (A), with a small amount of catalase present at the top of the gradient. In Group IV cells (B), catalase is present exclusively at the top of the gradient. Immunoblotting of group IV subcellular fractions (C) shows that precursor thiolase and 69-kD PMP peak in fractions 11 and 12.

only if Triton X-100 was included in the permeabilization. The ability to detect a particulate staining pattern for prethiolase-CAT and endogenous thiolase only when the peroxisomal membrane is permeabilized indicates that the PTS2-containing proteins are present inside the membrane.

Group IV Ghosts Fractionate at Low Density

Several investigations have indicated that peroxisomal ghosts fractionate at a lower density than peroxisomes from control cells (Santos et al., 1988; Gartner et al., 1991; Van Roermund et al., 1991). Subcellular fractionation was performed

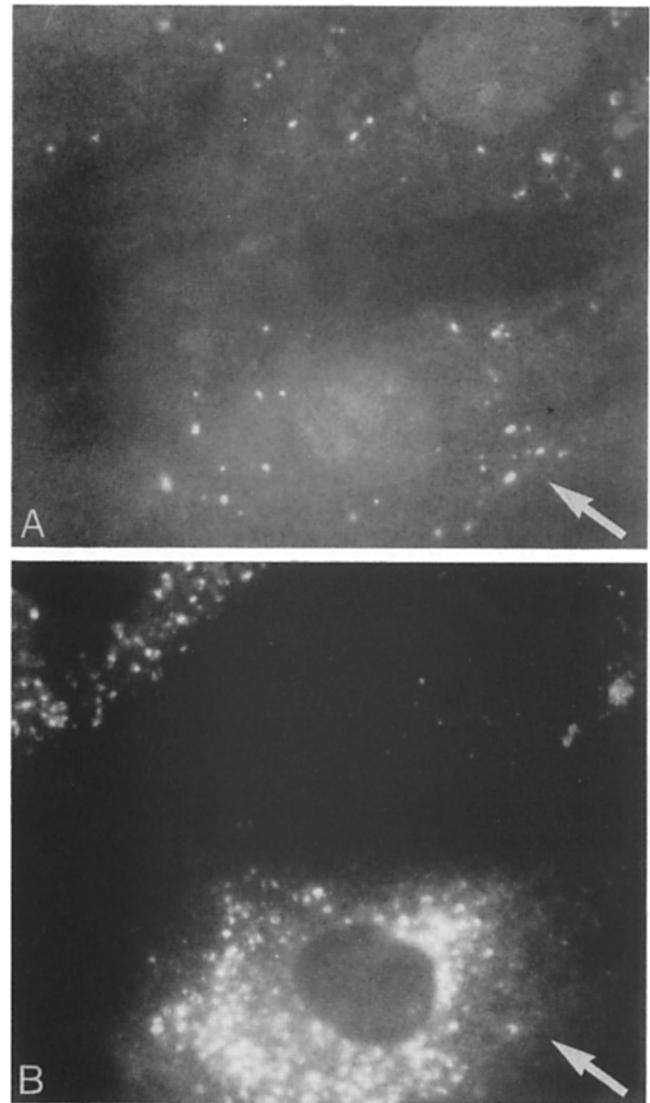
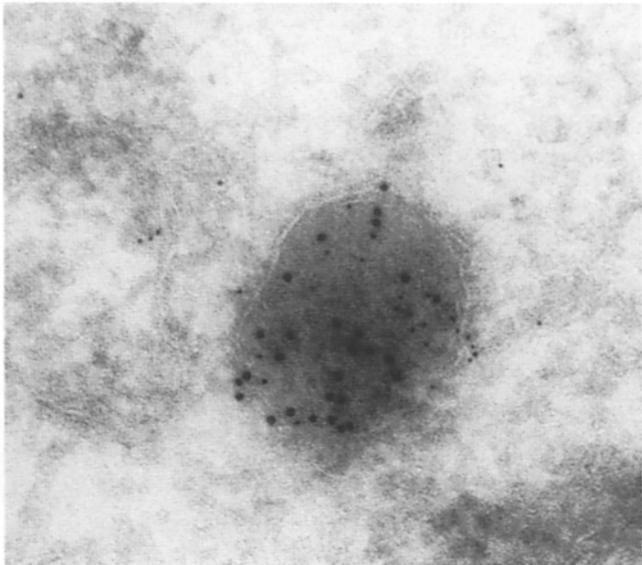
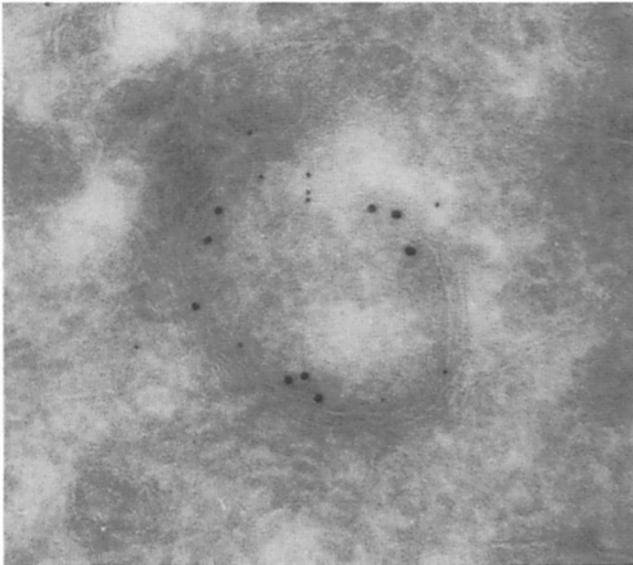
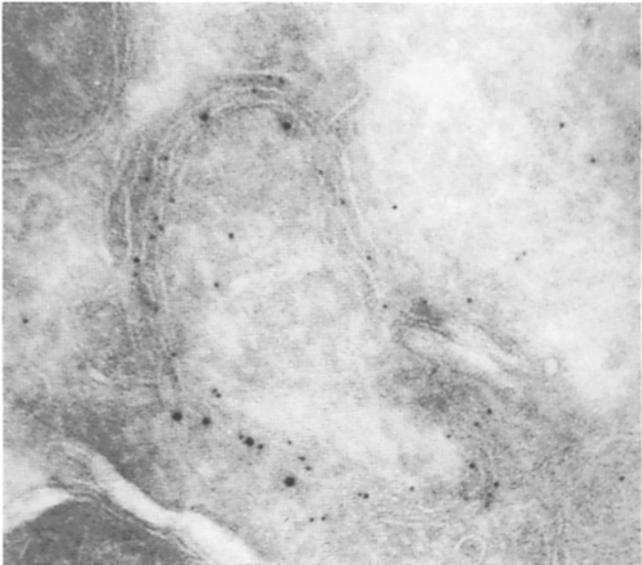
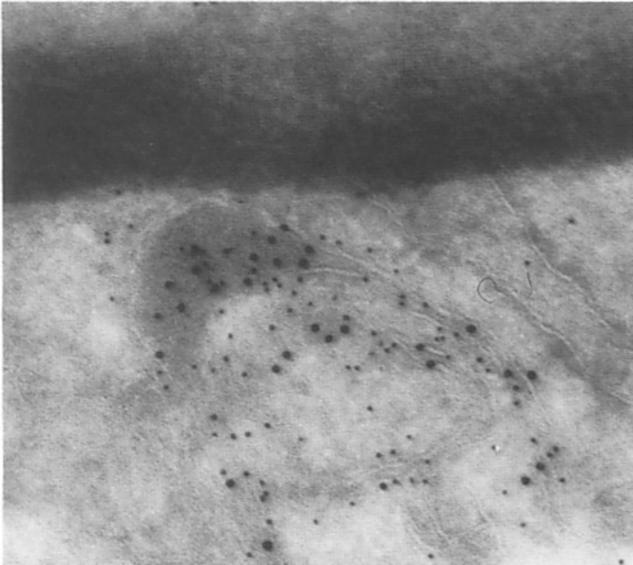
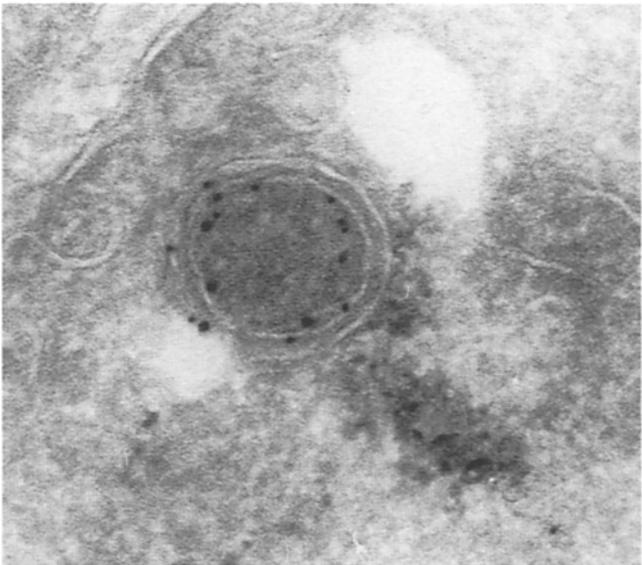
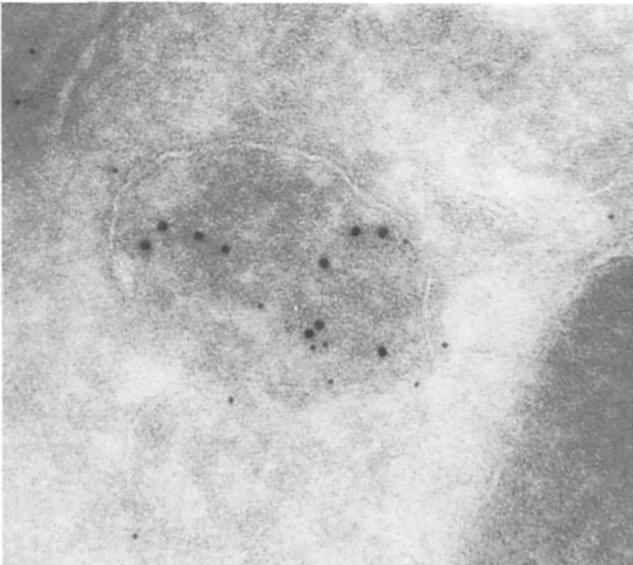


Figure 8. Most peroxisomal ghosts do not colocalize with lysosomes in group IV cells. Double-label immunofluorescence shows the subcellular localization of thiolase (A) and glucocerebrosidase (B) in group IV cells. Superimposition of the pattern of labeling in (A) onto that in (B) shows that most of the thiolase-containing particles do not colocalize with lysosomes. The arrow indicates an area in which there is clearly no overlap of these two compartments.

to investigate whether the prethiolase-containing particles in group IV cells also fractionate at a lower density than peroxisomes from control cells. Fig. 7 indicates that this was the case. In control cells, peroxisomes equilibrated in fractions 6 and 7, and they were well separated from the other organelle peaks (Fig. 7 A). In complementation group IV cells, however, catalase activity was found at the top of the gradient (Fig. 7 B), which is compatible with the diffuse pattern of labeling seen when catalase is visualized in group IV cells using immunofluorescence (Brul et al., 1988). In contrast to catalase, however, thiolase and the 69-kD PMP immunoreactivities migrated into the gradient and peaked in fractions 11 and 12 (Fig. 7 C), showing overlap with the lysosomal marker enzyme activity. Overlap with the lysosomal com-



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partment has been investigated in complementation groups II and III using double-label electron microscopy (Heikoop et al., 1992b). Heikoop et al. found that $\geq 80\%$ of the 69-kD PMP-containing peroxisomal ghosts showed labeling with anti- α -glucosidase and antiglucocerebrosidase, indicating that most of these structures are lysosomal compartments in groups II and III. We have investigated whether the thiolase labeling in group IV cells overlaps with that of the lysosomal enzyme glucocerebrosidase using double-labeling immunofluorescence. As shown in Fig. 8, the immunofluorescence staining patterns are strikingly different in general appearance, and most of the particles staining for thiolase (A) are clearly separated from the glucocerebrosidase-containing lysosomes (B). Santos et al. (1988b) also found that most of the peroxisomal ghosts do not colocalize with lysosomes in the Zellweger cell lines that they studied.

Thiolase Labeling is Associated with Multiple Membranes in Group IV Cells

Double-label electron microscopy was performed to investigate whether a membrane could be visualized associated with the thiolase labeling in group IV cells. The electron micrographs shown in Fig. 9 clearly indicate that thiolase labeling is enclosed by a membrane that contains the 69-kD PMP. Many of the residual "organelles" found were bounded by more than one membrane, and in these cases, the thiolase labeling is localized more to the periphery of the organelle. Several of the "organelles" appear to contain internal membranes, which in one example separate differences in electron density in the matrix. The appearance of these organelles is reminiscent of the thiolase-containing organelles found in the *S. cerevisiae* mutant *pas10* (Van der Leij et al., 1992).

Discussion

A promising line of research to gain insight into peroxisome biogenesis and the role of the components contributing to it is the characterization of cells impaired in the assembly of the organelle. Such cells are available in the form of fibroblasts derived from patients who suffer from peroxisome assembly disorders. We have studied the capacity of these fibroblasts to import PTS1- or PTS2-containing proteins into peroxisomes or residual peroxisomal structures by microinjection of plasmids encoding reporter proteins for each import pathway. Our results support the existence of differential import deficiencies: (a) loss of PTS1 import (complementation group IV); (b) loss of PTS2 import (group I); and (c) loss of both import routes (groups II, III, and V).

Loss of PTS1 import in complementation group IV, a "generalized" disorder of peroxisome assembly, is shown by the incompetence of the peroxisomal membranes in group IV cells to import luciferase produced from a microinjected expression plasmid. This result agrees with and extends the observation made by Walton et al. (1992) that microinjected

luciferase protein fails to be imported in two Zellweger cell lines. Notwithstanding the presence of ghostlike residual peroxisomal structures in group IV cells, which are generally considered to be import incompetent, we have found that the PTS2 import pathway is still functional. This is based on the following observations: (a) prethiolase-CAT gives rise to punctate fluorescence; (b) prethiolase-CAT can be unmasked to give punctate fluorescence only after permeabilization of the peroxisomal membrane in the presence of Triton X-100; (c) endogenous thiolase is protected from protease digestion in permeabilized group IV cells; and (d) prethiolase-CAT and endogenous thiolase colocalize with 69-kD PMP-containing membranes in group IV cells as shown by immunofluorescence, immunoelectron microscopy, and Nycodenz gradient subcellular fractionation. Furthermore, the possible presence of the bulk of the peroxisomal structures in group IV cells within lysosomes was excluded by the absence of colocalization with the lysosomal enzyme glucocerebrosidase. Taken together, these results indicate that group IV is a true differential import mutant. A similar phenotype has been demonstrated in the *S. cerevisiae* peroxisome assembly mutant *pas10*. In both cell types, peroxisomes are reduced to abnormal-looking membranous structures that are capable of importing thiolase but not SKL-containing proteins. The *PAS10* gene has been cloned by functional complementation of the *pas10* mutant (Van der Leij et al., 1993), and a homologue has been characterized from *P. pastoris* (McCollum et al., 1993). It encodes a protein of the tetratricopeptide repeat family that directly recognizes the SKL import motif (McCollum et al., 1993) (Franse, M. M., personal communication), supporting its direct role in the protein import process. Antibodies raised against the *S. cerevisiae* PAS10 protein recognize a specific peroxisomal protein from rat liver and human fibroblasts, and we are currently cloning the cDNA encoding this protein. This will enable us to test the interesting possibility of whether group IV cells can be complemented by expression of the PAS10p homologue.

Loss of PTS2 import is observed in complementation group I (RCDP) fibroblasts. This is based on (a) the lack of detectable import of prethiolase-CAT after expression of the PTS2 reporter protein in group I fibroblasts; and (b) the sensitivity of endogenous thiolase to protease digestion in permeabilized group I cells. The competence of the PTS1 pathway can be inferred from the equilibration of acyl-CoA oxidase (an SKL-containing enzyme) at normal density after subcellular fractionation of RCDP cells (Balfe et al., 1990), and it is confirmed by the appearance of punctate fluorescence after expression of luciferase in group I cells. The reason this differential protein import loss has not been noted before may be because of the finding by Balfe et al. (1990) that precursor thiolase present in low density fractions of an RCDP subcellular gradient is resistant to proteinase K digestion. In contrast, we have found that precursor thiolase is not protected from degradation by proteinase K in permeabilized RCDP (group I) cells. The clinical phenotype of RCDP is

Figure 9. Colocalization of 69-kD PMP and thiolase in aberrant membranous structures in group IV cells. 69-kD PMP, 5 nm gold; thiolase, 10 nm gold. The electron micrographs indicate that most of the structures found are larger than peroxisomes from control cells, and several of the structures contain multiple membranes.

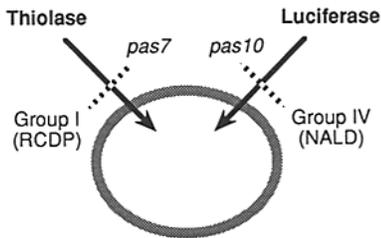


Figure 10. Human and *S. cerevisiae* cells deficient in peroxisome assembly display similar phenotypes. Peroxisomes in complementation group I and *S. cerevisiae pas7* are morphologically normal and are able to import PTS1-containing proteins but not PTS2-containing proteins into their matrix. In complementation group IV and *S. cerevisiae pas10*, atypical membranous structures are present that are able to import PTS2- but not PTS1-containing proteins. *NALD*, neonatal form of adrenoleukodystrophy.

thought to arise from an inability to synthesize plasmalogen: Wanders et al. have found a single enzyme deficiency of dihydroxyacetonephosphate acyltransferase (1992) or alkyldihydroxyacetonephosphate synthase (1993) in two RCDP patients. The finding of additional complementation groups amongst RCDP patients (Moser, A., personal communication) supports the view that several (at least four) genetic defects could give rise to impaired plasmalogen synthesis. The apparent inconsistency between our results and those of Balfe et al. may, therefore, result from the use of RCDP cells belonging to different complementation groups.

Again, the phenotype of the group I fibroblasts is strikingly similar to that of an *S. cerevisiae pas7* mutant. The peroxisomes present in both group I fibroblasts (Heikoop et al. 1991) and *S. cerevisiae pas7* cells (Van der Leij et al., 1992) are morphologically normal and are capable of importing PTS1-containing proteins and catalase but not thiolase into their matrix. The number of proteins entering the peroxisome via the PTS2 pathway appears to be rather limited compared to the number of proteins containing the PTS1. This may explain the difference in peroxisome morphology between human complementation group I (*S. cerevisiae pas7*) and group IV (*S. cerevisiae pas10*).

A generalized loss of import of PTS1- and PTS2-containing proteins is illustrated by groups II, III, and V. In these cells, only the routing of certain peroxisomal membrane proteins appears to be still functional. The phenotype of these cells may reflect a generalized impairment in the assembly or maturation of the organelle. Alternatively, by analogy with the import system for mitochondrial proteins, the defect may lie at a point at which the PTS1 and PTS2 import pathways converge, for example, at the protein translocation apparatus.

Complementation group V is a special case in this last category. The diffuse pattern of fluorescence found after expression of luciferase indicates the incapacity of the PTS1 import pathway. Expression of prethiolase-CAT, however, gives rise to a weak punctate fluorescence in a fraction of the microinjected cells. The reason for this intermediate phenotype is unclear. The mutation in Fujiki's group F has recently been shown to reside in the gene encoding the 35-kD PAF (peroxisome assembly factor) (Shimozawa et al., 1992). Remarkably, Shimozawa et al. have found an identical mutation in

PAF in our complementation group V (Shimozawa et al., 1993). This mutation introduces a stop codon, giving rise to a COOH-terminal truncated protein. The truncated PAF protein may show some residual activity. Another possibility is that prethiolase-CAT is targeted to ghosts in group V cells, but fails to be imported. The sensitivity of endogenous thiolase to proteinase K digestion in permeabilized group V cells supports this possibility.

In conclusion, we have found differential import deficiencies among the human peroxisome assembly disorders: the phenotypes found are analogous to phenotypes found amongst the *S. cerevisiae* peroxisome assembly mutants (see Fig. 10). The finding that differential protein import deficiencies also occur in the human peroxisome assembly disorders substantiates further that the mechanism of peroxisomal protein import is strongly conserved, not only in the use of similar targeting sequences, but also in the proteins comprising the import machinery.

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