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Peroxisomes in human fibroblasts have a basic pH.

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Peroxisomes are single membrane-bound organelles found in nearly all eukaryotic cells. These organelles have a central function in lipid metabolism, including the beta-oxidation of very-long and branched chain fatty acids and the biosynthesis of ether phospholipids and cholesterol. Another characteristic of peroxisomes is their ability to degrade hydrogen peroxide by catalase (1, 2). A deficiency in one or more peroxisomal enzymes has been linked to at least twenty (often lethal) disorders (3), showing the key role of this organelle in normal functioning of the human body. Peroxisomes are fragile structures that easily lose their integrity upon isolation (4). This poses a serious problem for studying these organelles *in vitro* and explains why our knowledge about the properties of the peroxisomal membrane including the (pH across it, is limited. *In vivo* peroxisomes have been shown to be closed structures that are impermeable to NAD(H) and NADP(H) implying the existence of NAD(P) redox shuttles (5,6). Here we study the pH in peroxisomes by targeting a pH-sensitive fluorescent reporter group to these organelles in living fibroblasts. We attained specific targeting by conjugating the fluorophore to a membrane-permeable peptide that contains a type I peroxisomal targeting sequence (PTS1; amino acid sequence AKL) (7). Using this peptide probe, we establish that peroxisomes of human fibroblasts have a pH of 8.2 ± 0.3. Fibroblasts from RCDP (rhizomelic form of chondrodysplasia punctata) type 1 patients with severe mutations in PEX7 protein, which result in an isolated defect in PTS2 protein import (8), are still capable of importing the probe into peroxisomes, but have a pH of 6.5 ± 0.3.

We covalently linked the pH sensitive (5-and 6-) carboxy-SNAFL-2 moiety to the PTS1-containing heptapeptide acetyl-CKGGAKL-COOH at the lysine near the amino-terminus. This peptide-probe (SNAFL-2-PTS1) was rapidly taken up into the cells and a punctate pattern of fluorescence was found, indicative of a peroxisomal localisation. To confirm that these structures are indeed peroxisomes, we used a fixable analogue (BODIPY-PTS1) in co-localization studies with Cy5-labelled antibodies against different peroxisomal proteins (figure 1a-c). The probe was targeted only towards the peroxisomes (figure 1b). Further evidence that the probe was incorporated into peroxisomes came from studies of human

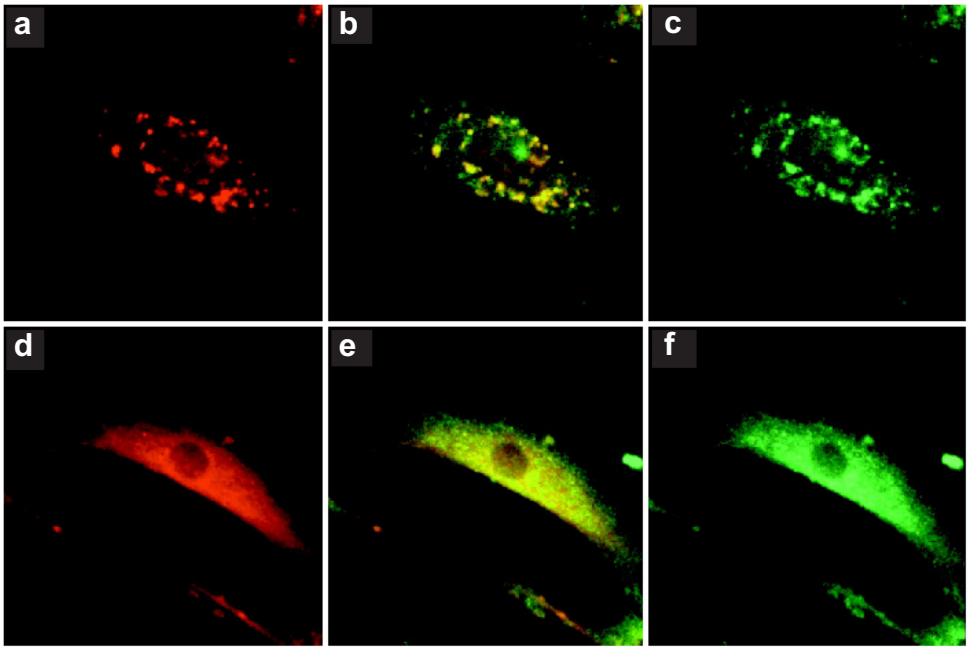

Figure 1. Peroxisomal localisation of BODIPY-PTS1. Incorporation of BODIPY-PTS1 in human fibroblasts (panel **a**). The high intensity structures coincide with that of an anti-nsL-TP-Cy5 staining (panel **c**) as becomes clear in the overlay **(b)**. Fibroblasts from a patient with a general assembly disorder (Zellweger) showed no structures with both the probe (panel **d**) and anti-nsL-TP-Cy5 (panels **e**,**f**). The same experiment carried out with Cy5-labelled antibodies against acyl-CoA oxidase, catalase and PMP70 gave similar results (data not shown). Colour-image at the back of this thesis.

fibroblasts with defects in peroxisomal import of PTS1-bearing proteins. This probe was not incorporated into peroxisomes in fibroblasts from a patient with mutations in PEX5(9) and an isolated defect in PTS1 protein import. We also failed to detect its incorporation into peroxisomes in cells from a PEX6-deficient patient with Zellweger syndrome who is unable to import PTS1- or PTS2-containing peroxisomal proteins (10). No or few peroxisomal structures were detected in these cells when using either the probe or the Cy5-labelled antibodies (figures 1d-f). ATP depletion, produced by treating cells with deoxyglucose, prevented the translocation of SNAFL-2-PTS1 to peroxisomes (data not shown); the probe remained diffusely distributed throughout the cell. This finding is in agreement with studies that showed that the targeting and incorporation of PTS1-containing proteins into peroxisomes is ATP-dependent (11).

The uptake of SNAFL-2-PTS1 by peroxisomes enables one to determine the pH of these organelles *in situ*. This probe (pKa of 7.7) consists of a basic form with a $\lambda_{em,max}$ of 625 nm and an acidic form with a $\lambda_{em,max}$ of 546 nm. These spectral properties are identical to those of the uncoupled SNAFL-2. By determining the ratio of the red and green fluorescence intensity by confocal laser scanning microscopy, and by comparing this ratio with a pH-calibration curve, the pH can be calculated. Figure 2b shows these ratios for SNAFL-2-PTS1 taken up by human fibroblasts and for unconjugated (5-and 6)-carboxy-SNAFL-2 in the buffer. Figure 2a shows the peroxisomal localization of the probe before addition of (5-and 6)-carboxy-SNAFL-2 to the buffer. The peroxisomal structures in figure 2a coincide with the structures having a lower acid:base ratio than the buffer (figure 2b). From comparison with the pH-calibration series (figure 2c, upper inset) it can be inferred that the pH of the

human fibroblast peroxisome is basic. We carried out identical ratio imaging experiments were carried out with fibroblasts from RCDP type 1 patients. The ratio image (figure 2c) shows no distinct structures although a clear peroxisomal localization of the SNAFL-2-PTS1 probe is observed (lower inset). Thus the pH in the peroxisomes (localization as outlined in figure 2c) is quite similar to that of the cytosol (i.e. pH 7.2).

The dissipation of the pH gradient in the RCDP fibroblasts must be a consequence of their mutations in PEX7 and the resulting defect in PTS2-protein import. RCDP cells are deficient in the known PTS2-targeted proteins alkyl-DHAP (dihydroxyacetone-phosphate) synthase and phytanoyl-CoA alpha hydroxylase and also in the PTS1 protein DHAP acyltransferase (3), the stability of which requires an interaction with alkyl-DHAP synthase (12). To determine whether the basic peroxisomal pH in wild-type cells is related to one of the enzymes deficient in the RCDP type 1 patients, we measured the pH in fibroblasts from patients with an isolated defect in DHAP acyltransferase (RCDP type 2(13)), alkyl-DHAP synthase (RCDP type 3 (14)) or phytanoyl-CoA alpha hydroxylase (Refsum disease (15)). All of these cells had a normal basic pH (data not shown). This indicates that the aberrant peroxisomal pH in RCDP type 1 cells must be due to a deficiency in some other enzyme that is not

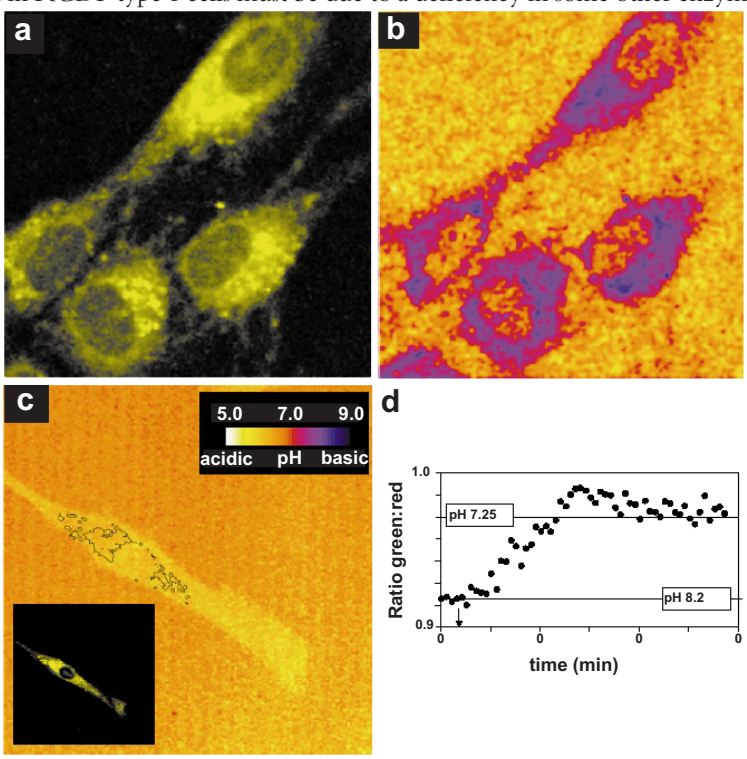

Figure 2. The peroxisomal pH in control and RCDP human fibroblasts. Control human fibroblasts were incubated with the pH-sensitive SNAFL-2-PTS1 (panel **a**). Free SNAFL-2 was added to the PBS++ and the ratio of the acidic and the basic form of the SNAFL-2 moiety both in the cell and in the PBS++ was measured. The peroxisomal structures in panel a coincide with the structures having a basic pH (panel **b**, and colour bar in panel **c**). The peroxisomes in fibroblasts from a patient with RCDP have a pH similar to that of the cytosol (panel **c**). The peroxisomal structures observed in the insert are outlined in black. Time-lapse ratio imaging after addition of the ionophore CCCP to control human fibroblasts was performed (panel **d**). Cells were labelled as described for panel **b**. Ratio images of the acidic over basic form of the SNAFL-2 moiety, both in the peroxisomes and in the PBS+ were taken every 30 sec. CCCP was added after 2 min (see arrow). For each image the acid/base ratio of the peroxisomes was normalised to that of the buffer (set at 1.00). Colour-image at the back of this thesis.

imported, destabilized or deprived of its substrate. The other possibility is that the mutations in PEX7, the PTS2 receptor, give rise to the formation of a transient pore in the peroxisome membrane, allowing protons to leak back into the peroxisome.

The origin of the basic pH in peroxisomes remains to be established. Depletion of ATP by deoxyglucose in human control fibrobalsts labelled with SNAFL-2-PTS1 did not result in a change in peroxisomal pH (data not shown), arguing against the involvement of an ATP-driven proton pump. On the other hand, addition of the uncoupler carbonyl *m*-chlorophenylhydrazone (0.5 μM) resulted in a complete dissipation of the gradient within 10 min (figure 2d). This indicates that the peroxisome membrane is impermeable to protons under normal conditions. The basic pH optimum and basic isoelectric point of most peroxisomal enzymes may reflect the adaptation to the alkalinity of the peroxisome.

Methods

Synthesis of the probes

The pH-sensitive SNAFL-2 was linked to one of the two lysines of the PTS1-peptide by incubating 1 mg acetyl-CKGGAKL-COOH (Ansynth Service BV Roosendaal, The Netherlands) and 0.5 mg carboxy-SNAFL-2-succinimidylester ((5-and 6-)-carboxy-seminaphtofluoresceine-2 succinimidylester, Molecular Probes) in 0.2 ml bicine buffer (100 mM bicine-NaOH, pH 8.4, 1:1 diluted with *N,N*-dimethylformamide) for 2 h at 37°C in the dark under continuous stirring. The product was precipitated by adding $CHCl_3$ / MeOH (4:1, v/v). Further purification of the peptide carrying the fluorophore on the lysine near the N-terminus was done by TLC and reversed phase HPLC on an Alltech C18 column (econosil, 250 mm). An analogous probe was synthesized containing the fluorophore BODIPY 530/550 IA (Molecular Probes) linked to cysteine group of the same heptapeptide. This probe was suitable for co-localization studies with Cy5-labelled antibodies in fixed cells.

Fluorescently labelled antibodies

Antibodies against the peroxisomal proteins acyl-CoA oxidase, catalase, PMP70, and non-specific lipid-transfer protein (nsL-TP) (purified by Dr. B.C. Ossendorp), were labelled with Cy5-sulfoindocyanine as described in Wouters *et al.* (16).

Tissue culture

Fibroblasts from patients with peroxisomal disorders and human control fibroblasts were cultured in HAM F-10 nutrient mix (Gibco) supplemented with 10% fetal calf serum and Penicillin/streptomycin (1%, Gibco), under a 5% CO_2 - atmosphere. The N-ALD patient with an isolated PTS1 import deficiency (complementation group 2 of the Kennedy Krieger Institute) has been described before (9). The Zellweger patient belonged to complementation group 4 (10). The RCDP patient was of type 1 (complementation group 11 of the Kennedy Krieger Institute) and was described before (8). The patients with dihydroxyacetonephosphate-acyltranserase deficiency (RCDP type 2)(13) and alkyl-dihydroxyacetonephosphate-synthase deficiency (RCDP type 3)(14) were described before.

Labelling of cells and confocal laser scanning microscopy

The medium of cells cultured on coverslips was replaced by enriched phosphate buffered saline (PBS++ :137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 1.5 mM

KH_2PO_4, 8.1 mM Na_2HPO_4 , supplemented with 5 mM glucose, pH 7.25) containing the PTS1-probes at a concentration of ± 5 μg/ml. After 1 h at 37°C the cells were rinsed with fresh PBS++, left for another 15 min and mounted in a temperature controlled lifechamber under the CLSM. Images were taken with a Leica TCSNT confocal laser-scanning system on an inverted microscope DMIRBE (Leica Microsystems, GmbH, Heidelberg, Germany) with an argon-krypton laser as excitation source. The BODIPY-PTS1 was excited with the 488 nm laserline and the emission was detected using a 530/30 bandpass filter. The green and the red fluorescence of SNAFL-2-PTS1 was acquired simultaneously using double wavelength excitation (laserlines 488 nm and the 568 nm) and emission (bandpass filters of 530/30 nm and 600/30 nm). Double labelling experiments were performed, using BODIPY-PTS1 and Cy5-labelled anti-Acyl-CoA oxidase, anti-PMP70, anti-catalase or anti-nsL-TP. After loading with the probe, the cells were fixed and permeabilized in PBS, pH 7.4 containing 3% paraformaldehyde, 0.2 % Triton X-100 and 0.2% glutaric aldehyde EM grade for 1 h at room temperature. The samples were further prepared according to ref. 16. The PTS1-probe in these samples was imaged as described above. The Cy5-labelled antibodies were excited with the 647 nm laserline and their emission was detected using a 665 longpass filter.

pH Measurements

pH-Calibration curves were acquired from free SNAFL-2 and from SNAFL-2-PTS1 diluted in buffers with pHs ranging from 4.0 to 9.0 using a Quantum Master spectrofluorometer (PTI, Surbiton, Surrey, UK). The pH-dependent fluorescence of SNAFL-2 was not affected by its linkage to the PTS1-peptide. Small glass capillaries filled with the calibration samples were used to acquire the pH-calibration curve on the confocal laser-scanning microscope. Images of SNAFL-2-PTS1 in cells were collected using the same microscope settings as were used for the calibration. The ratio of two fluorescence images recorded at 488/530 nm (acidic form of SNAFL2) and 568/600 nm (basic form of SNAFL2) was calculated on a Macintosh PowerPC computer using the NIH image program (W.Rasband). Free SNAFL-2 was added to the buffer as internal reference. The ratio image was filtered 5 times with the 'smooth' option of the NIH-Image program to reduce noise.

ATP-depletion and dissipation of proton gradients

Fibroblasts under the CLSM were treated with PBS+ (PBS++ without glucose) supplemented with 10 mM deoxyglucose for 15 min at 37°C to deplete ATP. Proton gradients were dissipated by addition of the ionophore carbonyl m-chlorophenylhydrazone (0.5 μM) to PBS+

Acknowledgments

We would like to thank Ms. P.A.W. Mooijer for her help with the tissue cultures.

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