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Localization of peroxisomal matrix proteins by photobleaching

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ABSTRACT

The distribution of some enzymes between peroxisomes and cytosol, or a dual localization in both these compartments, can be difficult to reconcile. We have used photobleaching in live cells expressing green fluorescent protein (GFP)-fusion proteins to show that imported bona fide peroxisomal matrix proteins are retained in the peroxisome. The high mobility of the GFP-fusion proteins in the cytosol and absence of peroxisomal escape makes it possible to eliminate the cytosolic fluorescence by photobleaching, to distinguish between exclusively cytosolic proteins and proteins that are also present at low levels in peroxisomes. Using this technique we found that GFP tagged bile acid-CoA:amino acid *N*-acyltransferase (BAAT) was exclusively localized in the cytosol in HeLa cells. We conclude that the cytosolic localization was due to its carboxyterminal non-consensus peroxisomal targeting signal (-SQL) since mutation of the -SQL to -SKL resulted in BAAT being efficiently imported into peroxisomes.

Introduction

Peroxisomes are small single membrane bound organelles that are present in almost all eukaryotic cells and are involved in fatty acid oxidation, synthesis of bile acids and plasmalogens and amino acid metabolism [1]. The peroxisomal matrix contains at least sixty different enzymes involved in these various metabolic pathways. A special feature of peroxisomal matrix proteins is that they are synthesized on free ribosomes and are imported into the peroxisomal matrix post-translationally and fully folded [2–4]. The majority of proteins destined for the peroxisomal matrix contain a carboxyterminal peroxisomal targeting signal 1 (PTS1), which binds to the import receptor peroxin-5 (PEX5), although a small number of proteins contain a PTS2, which interacts with peroxin-7 (PEX7). The precise intracellular distribution of some lipid metabolizing enzymes between cytosol and peroxisomes is often debated and in some instances difficult to unambiguously establish. Peroxisomes are notoriously "leaky" during cellular fractionation hampering biochemical analysis and using various techniques it is clear that some enzymes may localize in peroxisomes, the cytosol, or both, depending on the cell type. A dual localization is sometimes very difficult to detect in a background of high cytosolic levels using immunofluorescence microscopy. The differences in distribution pattern may result from differences in import efficiency. However, given the fact that peroxisomal proteins, like nuclear proteins, are imported without unfolding of the polypeptide chain and cleavage of the targeting signal, together with the fact that PEX7 was shown to shuttle in yeast [5], a dual localization of proteins could in principle be due to shuttling between the two compartments.

The PTS1 is defined as a tripeptide at the carboxyterminal of the protein. The first tripeptide identified was serine, lysine, leucine (-SKL) [6] and since then several variants of this consensus have been identified, (S/A/C), (R/K/H), (L/M), although predicting a functional PTS1 in silico can be difficult [7]. There are several proteins that contain non-consensus PTS1 tripeptides at their carboxyterminal end, which may result in a dual localization in peroxisomes and cytosol in some tissues. The human soluble epoxide hydrolase (hs-EH), which contains a PTS1 of -SKM, has a dual cytosolic and peroxisomal localization in vivo, which is cell specific in several tissues [8,9]. Interestingly, this protein is localized in peroxisomes in liver and kidney (although not exclusively), but is mainly, if not entirely, cytosolic in other tissues such as pancreatic islet cells, intestinal epithelium, anterior pituitary cells, adrenal gland, endometrium, lymphoid follicles, prostate ductal epithelium, alveolar wall, blood vessels and brain [9,10]. The biological significance of hs-EH is not fully understood, but substrates for the enzyme exist in both peroxisomes (isoprenoid phosphates) and cytosol (isoprenoid phosphates and fatty acid epoxides) [11]. The bile acid-CoA:amino acid *N*-acyltransferase (BAAT) catalyzes the final step in the production of conjugated bile acids in liver [12–14]. The intracellular localization of BAAT has been reported to be cytosolic, peroxisomal or microsomal in rat, mouse and human [14–20]. BAAT

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from all three species contain a carboxyterminal serine-glutamine-leucine sequence (-SQL), which is a variant of the PTS1 targeting signal of -SKL. Studies examining the intracellular localization of BAAT using green fluorescent fusion proteins showed that this fusion protein was entirely cytosolic in human skin fibroblasts [21], which was later confirmed in other studies [22]. However, in rat and human primary hepatocytes, BAAT was reported to be localized in peroxisomes [22], suggesting that targeting of non-consensus PTS1-containing proteins may be more efficient in primary liver cells than in cell lines and fibroblasts. The biological significance of a dual localization of BAAT in cytosol and peroxisomes has previously been discussed and it was suggested that BAAT is present in cytosol and peroxisomes to catalyze the conjugation of recycled or de novo synthesized bile acids, respectively [21,23,24]. Therefore a dual localization of BAAT may be required for de novo synthesis of bile acids in peroxisomes and in re-conjugation of recycled bile acids to glycine and/or taurine in the cytosol.

Using photobleaching techniques we now show that imported peroxisomal proteins are trapped inside the organelle and cannot escape back to the cytosol. We also demonstrate a procedure for photobleaching-assisted detection of low peroxisomal levels against a high cytosolic background. Using this technique we show that GFP-BAAT is exclusively localized in the cytosol in live HeLa cells and that this is likely a result of inefficient peroxisomal targeting mediated by its C-terminal -SQL sequence.

Materials and methods

Plasmids. Plasmids containing GFP-fusion proteins of wild-type human BAAT (hBAAT), hBAAT-SKL and ACOT8 were described previously [21,25]. The open reading frame of rat and mouse BAAT (rBAAT and mBAAT) were amplified by PCR using the following primers: rat BAAT 5′-ATGGCCAAGCTGACAGCTG-3′ and 5′-TCA CAGCTGACTGTTGAAACCTG-3′; mouse BAAT 5′-ATGGCCAAGCTGA CAGCTGTTCC-3′ and 5′-TCAGAGCTGACTGCTCAAATCTGG-3′. The PCR products were cloned into the pcDNA3.1/NT GFP (Invitrogen Corp.). Mutation of hBAAT-TSQL to -KSQL was carried out using Quikchange™ Site Directed Mutagenesis kit (Stratagene), using the following primers 5′-GCACCTCATTCCAGATGTG**AAA**AGTCAAC TCTAAGAAGACTAG-3′ (and the reverse complement primer), with the mutated codon underlined in bold. PCR was carried out for 16 cycles of 30 s at 95 °C, 1 min at 55 °C and 15 min at 68 °C. Mutations were verified by sequencing (Cybergene, Novum, Sweden).

Cell culture and transfections. HeLa cells (obtained from ATCC, CCL-2) and HepG2 cells were cultured in Eagle minimum essential medium (Sigma), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown on 0.17 mm glass coverslips at 37 °C in a humidified atmosphere containing 5% CO_2. For transient expression of plasmids, the FuGENE® HD Transfection Reagent was used (Roche) according to the Manufacturers instructions.

Confocal laser scanning microscopy (*CLSM*) *and photobleaching*. Live cells were monitored in phosphate buffered saline supplemented with 0.1% glucose, 24 h post-transfection. Imaging was performed on a Leica TCS-SP laser scanning confocal microscope (Leica, Heidelberg, Germany) with a 63.0 × 1.32 oil immersion objective using a 488 nm 20 mW argon laser line. Emission spectra were collected between 500 and 550 nm for detection of GFP. In Fluorescence Recovery After Photobleaching (FRAP) experiments, a defined region of a cell expressing GFP-ACOT8 or GFP-SKL was exposed to 100% laser intensity until the fluorescence was bleached out. Images were acquired at times indicated. For Fluorescence Loss In Photobleaching (FLIP) a defined region of cells expressing GFP-BAAT was repeatedly bleached for 1 s with 100% laser intensity and cells were imaged at low power between the bleach pulses.

Results and discussion

Peroxisomal matrix proteins are retained inside peroxisomes after import

In order to determine if imported peroxisomal matrix proteins become trapped or can shuttle back and forth between the organelle and the cytosol, we investigated the dynamic properties of peroxisomal proteins using FRAP (Fluorescence Recovery After Photobleaching) experiments on live cells in tissue culture. In FRAP experiments, recovery of fluorescence in peroxisomes in a bleached area of a cell and a corresponding decrease in peroxisome fluorescence outside the bleached area would indicate that peroxisomal proteins are able to shuttle back and forth between the organelle and the cytosol in analogy with nuclear proteins that undergo

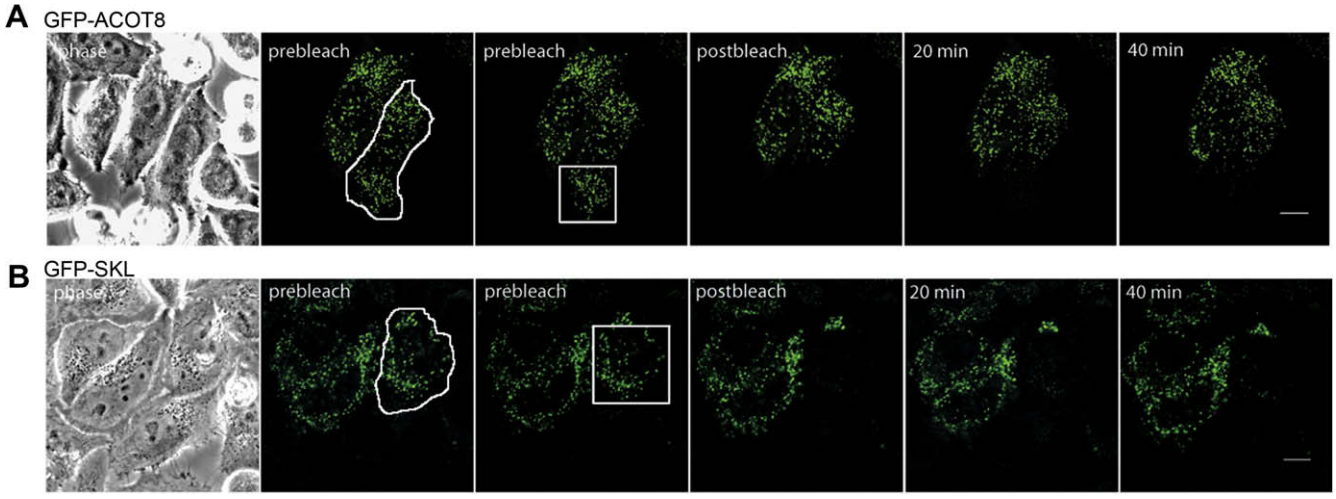

Fig. 1. Peroxisomal proteins are trapped inside peroxisomes. FRAP experiments on HeLa cells expressing GFP-ACOT8 (A) or GFP-SKL (B). A square in the lower part (A) or covering the major part (B) of one cell was completely bleached (white boxes). A white line delineates the cell contour. The neighboring unbleached cells serve as internal controls for unintentional bleaching during scanning. Corresponding phase contrast images are shown on the left. Images were acquired before (prebleach), directly after (postbleach), 20 and 40 min after bleaching. Note the lack of fluorescence recovery in the bleached area after 40 min in both cases. Scale bar, 10 μm.