The interaction between human PEX3 and PEX19 characterized by fluorescence resonance energy transfer (FRET) analysis

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The process of peroxisome biogenesis involves several \textit{PEX} genes that encode the machinery required to assemble the organelle. Among the corresponding peroxins the interaction between PEX3 and PEX19 is essential for early peroxisome biogenesis. However, the intracellular site of this protein interaction is still unclear. To address this question by fluorescence resonance energy transfer (FRET) analysis, we engineered the enhanced yellow fluorescent protein (EYFP) to the C-terminus of PEX3 and the enhanced cyan fluorescent protein (ECFP) to the N-terminus of PEX19. Functionality of the fusion proteins was shown by transfection of human \textit{PEX3}- and \textit{PEX19}-deficient fibroblasts from Zellweger patients with tagged versions of PEX3 and PEX19. This led to reformation of import-competent peroxisomes in both cell lines previously lacking detectable peroxisomal membrane structures. The interaction of PEX3-EYFP with ECFP-PEX19 in a \textit{PEX3}-deficient cell line during peroxisome biogenesis was visualized by FRET imaging. Although PEX19 was predominantly localized to the cytoplasma, the peroxisome was identified to be the main intracellular site of the PEX3-PEX19 interaction. Results were confirmed and quantified by donor fluorescence photobleaching experiments. PEX3 deletion proteins lacking the N-terminal peroxisomal targeting sequence (PEX3 34–373-EYFP) or the PEX19-binding domain located in the C-terminal half of the protein (PEX3 1–140-EYFP) did not show the characteristic peroxisomal localization of PEX3, but were mislocalized to the cytoplasm (PEX3 34–373-EYFP) or to the mitochondria (PEX3 1–140-EYFP) and did not interact with ECFP-PEX19. We suggest that FRET is a suitable tool to gain quantitative spatial information about the interaction of peroxins during the process of peroxisome biogenesis in single cells. These findings complement and extend data from conventional in vitro protein interaction assays and support the hypothesis of PEX3 being an anchor for PEX19 at the peroxisomal membrane.

Introduction

The biogenesis of peroxisomes in different species is a conserved process mediated by the concerted action of several proteins, termed peroxins (Distel et al., 1996). Among these, PEX3 and PEX19 are presumably involved in the very early stages of peroxisome assembly (Baerends et al., 1996; Snyder et al., 1999; Hettema et al., 2000). Human PEX3 is firmly anchored in the peroxisomal membrane with the C-terminus residing in the cytosol (Kammerer et al., 1998; Soukupova et al., 1999) and is thought to be required for the assembly of other membrane-bound peroxins in the peroxisomal membrane. \textit{PEX19} was initially described as a housekeeping gene (\textit{HK33}) (Braun et al., 1994) and was later shown to encode a farnesylated peroxisomal protein (Kammerer et al., 1997). However, the intracellular distribution of the PEX19 protein is predominantly cytoplasmic and partly peroxisomal and it interacts with a wide array of peroxisomal membrane proteins (Sacksteder et al., 2000; Snyder et al., 2000). Different hypotheses concerning the functional role of PEX19 are currently discussed. PEX19 may act as a soluble targeting receptor, which binds newly synthesized peroxisomal membrane proteins in the cytosol, targets these proteins to the peroxisome and facilitates their insertion into the peroxisomal membrane (Sacksteder et al., 2000). The observation, however, that PEX19 binds some
integral peroxisomal membrane proteins at regions distinct from their peroxisomal targeting sequence is not fully in line with this model (Fransen et al., 2001). From data in *Pichia pastoris* a chaperone-like role of Pex19p at the peroxisomal membrane is proposed. Under these circumstances, Pex19p would function as an assembly or disassembly factor or may regulate the formation of peroxin protein complexes in the peroxisomal membrane (Snyder et al., 2000).

Patients with a defect in peroxisome biogenesis display a continuum of severe clinical phenotypes and many of them die in early childhood (Gould et al., 2001). Defects in most PEX genes result in impaired peroxisomal matrix protein import, whereas the ability of membrane protein import is retained. This leads to the appearance of empty peroxisomal membrane "ghosts". Fibroblasts from patients with defects in PEX3, PEX16 or PEX19, however, display a cellular phenotype characterized by a lack of detectable residual peroxisomal structures, when investigated by indirect immunofluorescence analysis (Honsho et al., 1998; Matsuozono et al., 1999; South and Gould, 1999; Muntau et al., 2000; Sacksteder et al., 2000; Shimozawa et al., 2000). By contrast, in the yeast *Pichia pastoris* the application of sensitive techniques recently led to the detection of peroxisomal remnants in Apx3 and Apx19 cells (Hazra et al., 2002). The interaction of PEX3 and PEX19 is thought to be essential for early peroxisome membrane formation and has been the subject of several studies in yeast (Götte et al., 1998; Snyder et al., 1999) and in humans (Soukupova et al., 1999; South and Gould, 1999; Muntau et al., 2000, Fransen et al., 2001). However, the intracellular site of the interaction between PEX3 and PEX19 during the complex process of peroxisome biogenesis is still unclear.

A powerful technique to study the intracellular localization of intermolecular protein interactions in living cells is fluorescence resonance energy transfer (FRET). FRET microscopy has been applied to analyze the function of reporter constructs (Miyawaki et al., 1997), the organization of supramolecular complexes such as SNARE complexes (Xia et al., 2001), to investigate receptor oligomerization (Siegel et al., 2000), and protein phosphorylation states (Ng et al., 1999), to characterize membrane microdomain structures (Simons and Toomre, 2000) and, most recently, the topographical features at an intermembrane junction (Wong and Groves, 2002). In peroxisomal biology FRET analysis has so far only been used to study the association of non-specific lipid transfer protein with fatty oxidation enzymes in peroxisomes (Wouters et al., 1998). FRET is a spectroscopic technique used for quantifying the distance between two molecules conjugated to differently colored fluorophores with Ångström precision (Clegg, 1992). FRET microscopy can distinguish proteins that are merely undergoing protein-protein interactions (Lippincott-Schwartz et al., 2001). It involves the transfer of photon energy from a fluorescent donor in its excited state to another excitable moiety, the acceptor, by a nonradiative dipole-dipole interaction (Lakowicz, 1999), resulting in emission of the characteristic acceptor fluorescence. The FRET efficiency dramatically decreases when the distance between the donor and the acceptor increases. However, steady-state FRET microscopy can suffer from several sources of distortion. It depends on the extent of spectral overlap between the donor emission and acceptor excitation, the quantum yield of the donor, and on the relative orientation of the donor and acceptor. For reliable results, appropriate control experiments have to be performed (Lippincott-Schwartz et al., 2001). The enhanced variants of the green fluorescent protein (EGFP), cyan fluorescent protein (ECFP) and yellow fluorescent protein (EYFP), have been shown to be a suitable FRET pair to study protein interactions in vivo (Heim and Tsien, 1996; Miyawaki et al., 1997; Llopis et al., 2000; Sorkin et al., 2000; Janetopoulos et al., 2001; Schmid et al., 2001; Wilson et al., 2002). In this study we used the ECFP-EYFP donor-acceptor pair for FRET imaging to characterize the intracellular site of interaction between PEX3-EYFP and ECFP-PEX19 in single PEX3-deficient cells during peroxisomal reconstitution. Results were confirmed and quantified by donor fluorescence photobleaching experiments. The consequences of a disruption of the peroxisomal targeting sequence or the PEX19-binding domain on the protein-protein interaction were studied using PEX3 deletion constructs.

### Materials and methods

#### Plasmid construction

To generate plasmids encoding PEX3-EYFP and PEX3-ECFP, the cDNA for EGFP was removed from pMS204 (encoding PEX3-EGFP) (Soukupova et al., 1999) by restriction digestion with enzymes ApaI and NotI and substituted by the sequence for EYFP and ECFP derived from pEYFP-N1 and pECFP-N1 (Clontech) by restriction digestion with enzymes ApaI and NotI.

To generate the plasmids coding for ECFP-PEX19 and EYFP-PEX19, the sequences for ECFP and EYFP were PCR-amplified using pECFP-N1 and pEYFP-N1 as a template and primers XFP-HindIII-fo (5'-cgagatattacagctcgtcc-3') and XFP-BamHI-rev (5'-gccgtacctgtaacgctc-3'), respectively. The corresponding fragments were digested with HindIII and BamHI restriction endonucleases and were subsequently ligated into the corresponding sites of full-length PEX19 cDNA in the expression plasmid HsPxFall (Kammerer et al., 1997). Plasmids encoding an N-terminally truncated form of PEX3 fused to EYFP, namely PEX3 34–373-EYFP, and a C-terminally truncated form of PEX3 fused to EYFP, namely PEX3 1–140-EYFP, were generated using the plasmids pMS218 and pMS203, respectively (Soukupova et al., 1999), and substituting EGFP by EYFP as described above.

#### Cell culture and transfection

The human PEX3-deficient skin fibroblast cell lines and the human PEX19-deficient cell lines were transfected with the large T-antigen of SV40 virus as described (Dodt et al., 1995). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 000 U/l penicillin, and 100 mg/l streptomycin at 9% CO₂.

All transfections of human cell lines were performed using Lipofectamine (Life Technologies) according to the manufacturer’s instructions. Three or five days after transfection the cells were processed for immunofluorescence microscopy according to Slawecki et al. (1995), but cells were mounted on glass slides using 10 μl of a Mowiol-based mounting solution containing 2.5 mg/ml n-propylgallate (Hille et al., 1992). If indicated, antibodies against PEX14 were used (1:400), followed by incubation with Alexa 568- or Alexa 594-conjugated secondary antibodies.

For functional complementation analysis, PEX3-deficient cell lines were transfected with plasmids encoding PEX3-EYFP and PEX3-ECFP, while PEX19-deficient cell lines were transfected with plasmids for EYFP-PEX19 and ECFP-PEX19, respectively. Three days after transfection cells were fixed, mounted and investigated by confocal laser scanning microscopy (CLSM; Zeiss LSM 510) as outlined below.
FRET microscopy (imaging and donor photobleaching FRET microscopy)

Fluorescence images of cells were acquired on a confocal laser scanning microscope. For fluorescence detection of the donor ECFP cells were excited with a 458 nm Ar laser and examined using a 540–600 nm band pass filter (“donor setting”). For detection of the acceptor EYFP, cells were excited with a 514 nm Ar laser and viewed using a 530–600 nm band pass filter (“acceptor setting”). For detection of FRET, cells were excited with a 458 nm Ar laser and analysed using a 530–600 nm band pass filter (“FRET setting”). Images were captured with all settings under identical conditions. Cells were imaged on an Axiovert 135TV microscope (Zeiss) using a 1.4 numerical aperture 63× Zeiss Plan-Apochromat objective and processed using LSM 510 Software (Zeiss).

For quantitation of FRET, donor fluorescence photobleaching FRET microscopy was performed by bleaching a defined region of 45 μm² with four iterations (pixeltime 12.5 ps) using the ECFP excitation wavelength. After each bleaching procedure the region of interest was scanned to generate an image. In total, 20 images were acquired. Regions of interest were selected over the cytosol and peroxisomes, and fluorescence emission intensities were quantified using Prism 3.0 (GraphPad Software, San Diego). The resulting decay curves were fitted to the equation for a single exponential decay approaching a constant value: fluorescence intensity = A₀ e⁻ᴷᵗ + offset, where A₀ denotes the starting value, offset denotes the final fluorescence signal, K is the decay constant and t denotes a relative value defined by iterations of bleaching procedures. The relative fluorescence half-time is defined to be 1/K·0.697.

Statistical analysis

Statistical analysis was performed using Prism 3.0 (GraphPad Software, San Diego). The significance of differences among the means of various groups was determined by Student’s t test for independent samples.

Results

EYFP- and ECFP-tagged versions of PEX3 and PEX19 restore peroxisome biogenesis in PEX3- and PEX19-deficient cell lines

We used human PEX3- and PEX19-deficient cell lines to test the functionality of the EYFP- and ECFP-tagged PEX3 and PEX19 proteins. Both cell lines are derived from patients with severe forms of Zellweger syndrome and entirely lack detectable peroxisomal membrane structures, when analyzed by indirect immunofluorescence (Matsuzono et al., 1999; Muntau et al., 2000). We fused the yellow fluorescent protein EYFP to the C-terminus of PEX3 and the cyan fluorescent protein ECFP to the N-terminus of PEX19 in order not to hamper the N-terminal peroxisomal targeting sequence of PEX3 or the C-terminal farnesylation sequence of PEX19. Transfection rates showed only minor differences (PEX3-EYFP, 5.5%; ECFP-PEX19, 4.9%) to those obtained with plasmids encoding wild-type PEX3 (5.5%) or PEX19 (6.7%). Transfection of the PEX3-deficient cell line with a plasmid encoding PEX3-EYFP led to a punctate pattern indicating that these proteins were predominantly localized to peroxisomes (Fig. 1a). Transfection of the PEX19-deficient cell line with the cDNA coding for ECFP-PEX19 yielded a diffuse staining, demonstrating that most of this protein was expressed in the cytoplasm and considerable amounts of ECFP-PEX19 were found in the nucleus (Fig. 1b). In few cells, however, ECFP-PEX19 was localized to peroxisomes (data not shown). Transfection of all plasmids encoding PEX3- and PEX19-fluorescence proteins led to reformation of import-competent peroxisomes as evidenced by colocalization with PEX14 (Fig. 1a, b) and catalase (data not shown). These results indicated that all constructs were fully functional.

Intracellular localization of PEX3 deletion constructs

In addition to full-length PEX3-EYFP we investigated interaction of two PEX3 deletion proteins with ECFP-PEX19 by FRET analysis in PEX3-deficient fibroblasts. The first one lacked the N-terminal 33 amino acids corresponding to the peroxisomal targeting sequence (PEX3 34–373-EYFP), the second one lacked the C-terminal half of the protein containing the PEX3-PEX19 interacting region recently mapped to amino acids 148 to 307 (PEX3 1–140-EYFP) (Franzen et al., 2001). In contrast to full-length PEX3-EYFP, which was located at peroxisomes, transfection of PEX3-deficient fibroblasts with plasmids for PEX3 34–373-EYFP and PEX3 1–140-EYFP revealed mislocalization of these two proteins: PEX3 34–373-EYFP to the cytoplasm and PEX3 1–140-EYFP to the mitochondria (Fig. 2) as evidenced by colocalization with a mitochondrial marker (MitoTracker, Molecular Probes). Transfection rates were comparable (PEX3 34–373-EYFP, 7.5%) or even higher (PEX3 1–140-EYFP, 11.0%) than those obtained with wild-type constructs (6.3%). In contrast to transfection of full-length PEX3-EYFP cDNA, transfection of the PEX3 deletion constructs did not lead to restoration of peroxisomes indicating that these constructs were not functional in peroxisomal biogenesis.

The interaction between PEX3-EYFP and ECFP-PEX19 visualized by FRET imaging

The fluorophores ECFP and EYFP can be used for FRET analysis because the emission spectrum of ECFP (donor molecule) overlaps the excitation spectrum of EYFP (acceptor molecule) (Fig. 3). R₀ (critical Förster radius) is defined as the distance at which FRET efficiency is 50%. The R₀ for ECFP-PEX19 is approximately 50 Å (5 nm), the largest value of all known donor-acceptor pairs. When the distance between ECFP and EYFP fusion proteins is close, e.g. upon physical interaction, excitation of the donor (ECFP) at 458 nm leads to nonradiative energy transfer from the donor to the acceptor (EYFP) and light at a wavelength of 530–600 nm within the emission spectrum of the acceptor is released (Fig. 3).

All FRET experiments were performed in a PEX3-deficient cell line assuming that the PEX3-PEX19 interaction is functionally relevant especially for early peroxisome assembly and therefore easier to detect during peroxisomal reconstitution rather than in wild-type cells. Three days after co-transfection of ECFP-PEX19 and PEX3-EYFP the donor ECFP-PEX19 was mainly expressed in the cytoplasm with only little signal also emitted at the peroxisome as shown in images acquired by excitation and detection of the donor (“donor setting”) (Fig. 4a). The acceptor PEX3-EYFP was mainly expressed at peroxisomes as shown by colocalization of structures acquired by excitation and detection of the acceptor (“acceptor setting”) and of the peroxisomal membrane protein PEX14 (Fig. 4a, b). Most of the FRET signal detected was found at peroxisomes as shown in images acquired by excitation of the donor and detection of the acceptor (“FRET setting”). Since the acceptor itself was not excited, most of this signal identified was due to energy transfer from ECFP to EYFP and thus to physical interaction between ECFP-PEX19 and PEX3-EYFP at the peroxisome. Some FRET signal was detected also in the cytoplasm (Fig. 4a). In rare instances, where both ECFP-
PEX19 and PEX3-EYFP were expressed at the peroxisome, the resulting FRET signal was particularly strong (Fig. 4b). Co-transfection of the empty vector ECFP expressed in the cytosol and PEX3-EYFP mainly expressed at the peroxisome was used as a control experiment. Only a weak background signal was identified using the FRET setting suggesting that no physical interaction between ECFP and PEX3-EYFP had occurred (Fig. 5a).

However, a number of factors such as cross talk, background fluorescence and the fact that each of the fluorescence components identified consists of both FRET as well as non-FRET components may influence FRET imaging (Kenworthy, 2001). The degree of fluorescence overflow in our experimental setting can be estimated by transfection of a PEX3-deficient cell line with plasmids encoding either ECFP-PEX19 (Fig. 5b) or PEX3-EYFP (Fig. 5c) separately and by subsequent immunofluorescence analysis using the donor setting and the FRET setting (Fig. 5b) or the acceptor setting and the FRET setting (Fig. 5c), respectively. A method to correct for the factors mentioned above would be the one proposed by Gordon et al. (1998), which requires elaborate calculations. Although reliable, this method is not suitable for high-throughput analysis. To confirm our FRET imaging results, we therefore decided to perform donor fluorescence photobleaching experiments that also allow for quantitative analysis. This method has the additional advantage that it is independent of fluorophore concentration and is not affected by differences in intracellular distribution of donor and acceptor.

Quantitation of FRET by donor fluorescence photobleaching

Donor fluorescence photobleaching relies on the fact that bleaching of a donor fluorophore is slower in the presence of a FRET acceptor because energy is transferred from the donor to the acceptor and thus is unavailable for bleaching the donor. The time constant of the fluorescence decay is independent of
fluorophore concentration as long as the acceptor is present in sufficient amounts. Defined regions (45 μm²) in PEX3-deficient cells co-expressing ECFP-PEX19 and PEX3-EYFP, PEX3 34–373-EYFP, or PEX3 1–140-EYFP, respectively, were bleached 20 times (with 4 iterations each) using the donor excitation wavelength of 458 nm. This led to a decline of donor fluorescence emission in a monoexponential manner (Fig. 6a). When analyzing the interaction of full-length PEX3-EYFP with ECFP-PEX19 or with ECFP, the relative donor fluorescence half-time was significantly higher in cells co-expressing ECFP-PEX19 and full-length PEX3-EYFP than in cells co-expressing the empty vector ECFP and PEX3-EYFP (Fig. 6b).

These results are in agreement with those obtained by FRET imaging and indicate that FRET and thus physical interaction occurred between PEX3-EYFP and ECFP-PEX19 but not between PEX3-EYFP and ECFP.

In analyzing the interaction of the N-terminal PEX3 deletion protein PEX3 34–373-EYFP with ECFP-PEX19 or with ECFP we found no significant difference in the relative donor fluorescence half-time upon donor photobleaching between cells co-expressing ECFP-PEX19 and PEX3 34–373-EYFP and in cells co-expressing ECFP and PEX3 34–373-EYFP (Fig. 7). These results indicate that FRET did not occur between PEX3 34–373-EYFP and ECFP-PEX19 and between

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**Fig. 2.** Intracellular localization of PEX3 deletion constructs fused to EYFP compared to full-length PEX3-EYFP after transfection into a PEX3-deficient cell line. Expression of PEX3-EYFP yielded a punctate pattern indicating that full-length PEX3-EYFP was located at peroxosomes (left panel), whereas transfection of PEX3 34–373-EYFP led to a diffuse staining indicating cytosolic localization (central panel). After expression of PEX3 1–140-EYFP a tubular pattern indicated localization of PEX3 1–140-EYFP at the mitochondria (right panel).

**Fig. 3.** The principle of FRET. The excitation (dashed lines) and emission (continuous lines) spectra of ECFP and EYFP are shown in dark and light gray, respectively (a). The emission spectrum of ECFP (donor) overlaps the excitation spectrum of EYFP (acceptor). For FRET analysis, ECFP was fused to the N-terminus of PEX19 and EYFP was fused to the C-terminus of PEX3 (b). If the distance between the donor molecule (ECFP) and the acceptor molecule (EYFP) is small (1–5 nm, 10–50 Å), excitation of the donor at a wavelength of 458 nm leads to nonradiative energy transfer from the donor to the acceptor, and light with the emission spectrum of the acceptor at 530–600 nm is emitted.
PEX3 34–373-EYFP and ECFP and thus PEX3 34–373-EYFP did not interact with ECFP-PEX19. In this experiment the two proteins were located in the same subcellular compartment, the cytoplasm. This resulted in a somewhat higher background signal as shown by comparison of the mean relative fluorescence half-times (Tab. I).

Similar results were obtained when the interaction of the C-terminal PEX3 deletion protein PEX3 1–140-EYFP with ECFP-PEX19 or with ECFP was analyzed. We identified no significant difference in the relative donor fluorescence half-time upon donor photobleaching between cells co-expressing ECFP-PEX19 and PEX3 1–140-EYFP and in cells co-expressing ECFP and PEX3 1–140-EYFP, with PEX3 1–140-EYFP being mislocalized to the mitochondria (Fig. 7). These results indicate that FRET did not occur between PEX3 1–140-EYFP and ECFP-PEX19 and between PEX3 1–140-EYFP and ECFP and thus PEX3 1–140-EYFP did not interact with ECFP-PEX19. The mean relative donor fluorescence half-times obtained after co-transfection of different constructs are summarized in Table I.

### Discussion

The process of peroxisomal biogenesis involves at least 24 PEX genes that encode the machinery required to assemble the organelle (Purdue and Lazarow, 2001). Although the progress in the field has been significant in the last years, our knowledge about spatial and temporal aspects of the interaction of different peroxins during peroxisome assembly still remains limited. Here we present several lines of evidence that FRET microscopy is a suitable tool to study the localization of peroxin interactions in single living cells during peroxisome assembly.

First, we showed that EYFP- and ECFP-tagged versions of PEX3 and PEX19 were fully functional by demonstrating their ability to restore peroxisome biogenesis in PEX3- and PEX19-deficient human cell lines from patients suffering from Zellweger syndrome. Second, analysis of the interaction between

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**Tab. I.** Mean relative donor fluorescence half-times obtained by donor fluorescence photobleaching after co-transfection of different combinations of expression plasmids.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Mean relative donor fluorescence half-time</th>
<th>SEM</th>
<th>n</th>
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<tbody>
<tr>
<td>PEX3-EYFP/ECFP-PEX19</td>
<td>2.92</td>
<td>0.13</td>
<td>22</td>
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<tr>
<td>PEX3-EYFP/ECFP</td>
<td>1.89</td>
<td>0.11</td>
<td>17</td>
</tr>
<tr>
<td>PEX3 34-373-EYFP/ECFP-PEX19</td>
<td>2.50</td>
<td>0.12</td>
<td>19</td>
</tr>
<tr>
<td>PEX3 34-373-EYFP/ECFP</td>
<td>2.50</td>
<td>0.18</td>
<td>13</td>
</tr>
<tr>
<td>PEX3 1-140-EYFP/ECFP-PEX19</td>
<td>2.39</td>
<td>0.09</td>
<td>17</td>
</tr>
<tr>
<td>PEX3 1-140-EYFP/ECFP</td>
<td>2.44</td>
<td>0.14</td>
<td>13</td>
</tr>
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SEM, standard error of mean; n, number of experiments performed.
Finally, we demonstrated that two PEX3 deletion proteins upon PEX3-PEX19 interaction during peroxisomal biogenesis. To confirm our results and to quantitate FRET occurring, we applied the donor fluorescence photobleaching method as a tool to determine the interaction between PEX3 and PEX19 in vivo. Third, we demonstrated that the peroxisome is the main intracellular site of this interaction during peroxisomal biogenesis in human PEX3-deficient fibroblasts provided first strong evidence that the peroxisome is the main intracellular site of this interaction during peroxisomal biogenesis in vivo. Third, we applied the donor fluorescence photobleaching method as a tool to confirm our results and to quantitate FRET occurring upon PEX3-PEX19 interaction during peroxisomal biogenesis. Finally, we demonstrated that two PEX3 deletion proteins fused to EYFP were mislocalized to the cytosol or to the mitochondria and did not interact with PEX19 using the methods applied in this study.

Previous studies have shown that human PEX19 is bimodally distributed between the cytoplasm and the peroxisomal membrane, with more than 95% of PEX19 being cytoplasmic (Sacksteder et al., 2000). Nevertheless, in our protein interaction studies most of the FRET signal was detected at peroxisomes with little signal identified in the cytoplasm. These results obtained in live human cells are in agreement with previous data from cross-linking and subcellular fractionation experiments suggesting that the peroxisome, and not the cytoplasm, is the steady-state site of interaction between Pex19p and Pex3p in Pichia pastoris (Snyder et al., 2000). PEX19 is known to bind several peroxisomal membrane proteins in the cytosol (Sacksteder et al., 2000), which are subsequently targeted to the peroxisomal membrane. Our observations support the hypothesis that PEX3 may function as a peroxisomal membrane protein anchor for PEX19 integrating other peroxisomal membrane proteins into the membrane of the organelle. Binding of PEX3 to PEX19 in the cytoplasm and PEX3 being thereby translocated to the peroxisome appears less likely, based on our results. Data showing that PEX19 contains distinct, non-overlapping binding sites for PEX3, PEX16, and the group of membrane proteins PEX10, PEX11, PEX12, and PEX13 (Fransen et al., 2001) may be in line with this hypothesis.

The detection of some FRET signal in the cytoplasm may suggest that PEX3 and PEX19 can in principle also interact in the cytoplasm, at least under artificial in vitro conditions after overexpression or mislocalization of donor or acceptor proteins. Sacksteder et al. showed that in wild-type human fibroblasts mistargeting of PEX19 to the nucleus leads to nuclear accumulation of PEX3, implying that binding of PEX3 by PEX19 may have previously occurred in the cytoplasm (Sacksteder et al., 2000). Other in vitro data using immunoprecipitations from cell-free extracts and two-hybrid data obtained both with the yeast and the human system also demonstrate that the full-length proteins are capable to interact in solution (Soukupova et al., 1999; Fransen et al., 2001).

We subsequently investigated whether either a lack of the peroxisomal targeting sequence or of the PEX19-binding domain of PEX3 influence the interaction between PEX3 and PEX19. For this purpose, we used two different PEX3 deletion constructs. The peroxisomal targeting sequence of human PEX3 was previously reported to be contained within its N-terminal 33 (Soukupova et al., 1999) or 45 amino acids (Kammerer et al., 1998; Fransen et al., 2001). Under the in vivo conditions applied in our study a lack of the PEX3 peroxisomal targeting sequence abolished the interaction with full-length ECFP-PEX19 as suggested by the fact that the mean relative fluorescence half-time in the donor fluorescence photobleaching experiments did not differ from controls utilizing ECFP. The finding that no significant FRET took place between PEX3 34–373-EYFP and ECFP-PEX19 both being located to the cytoplasm confirms that FRET microscopy yields specific results and that colocalization of two proteins in the same compartment at the level of light microscopy does not necessarily lead to the detection of a significant FRET signal. However, previous in vitro studies reported an interaction between N-terminal PEX3 deletion constructs and PEX19 using the two-hybrid system (Fransen et al., 2001) or in vitro binding assays (Mayerhofer et al., 2002). Since in this study the

**Fig. 5.** Lack of FRET after co-transfection of ECFP and PEX3-EYFP (a). As a control, the empty vector ECFP and PEX3-EYFP were co-transfected in a PEX3-deficient cell line, and cells were processed for FRET analysis 3 days later. Transfection of ECFP led to a diffuse cytosolic and nuclear signal (upper left panel), whereas transfection of the acceptor PEX3-EYFP led to a punctate pattern indicating its expression at the peroxisome (lower left panel) as demonstrated by colocalization with the peroxisomal membrane protein PEX14 (lower right panel). In the absence of PEX19 only a weak signal was detected using the FRET setting (upper right panel) indicating a lack of physical interaction between PEX3-EYFP and ECFP. Control experiments to estimate fluorescence overflow (b, c). Expression of ECFP-PEX19 separately (b) and analysis using the donor setting (left panel) showed an intense signal, whereas application of the FRET setting (right panel) revealed a weak signal due to fluorescence overflow from the CFP to the YFP channel. Expression of PEX3-EYFP separately (c) and analysis using the acceptor setting (left panel) gave an intense punctate signal, whereas application of the FRET setting (right panel) showed almost no signal.
Peroxisomal membrane has been identified to be the main site of the PEX3-PEX19 interaction, the intracellular mislocalization of PEX3 34–373-EYFP from the peroxisome to the cytoplasm may explain these discrepancies, in that mislocalization may lead to misfolding and low binding affinity of the PEX3 34–373-EYFP protein to PEX19. Although unlikely, we cannot totally exclude the possibility of this result being a “false negative” one. In this case, the N-terminally truncated PEX3 protein, which in vivo would be mislocalized to the cytosol, would be able to interact with PEX19, as previously shown by transfection of the empty vector ECFP with PEX3-EYFP (●, n = 17, FRET negative). This slower bleaching of the donor fluorophore in comparison to the control indicated that FRET had occurred. Relative donor fluorescence half-times of cells transfected with the indicated combinations of expression plasmids (b). The boxes represent the 50 percent confidence interval (25th – 75th percentile); the horizontal black bars represent the medians; the error bars indicate the minimum-maximum range. The p value refers to the difference between the mean donor fluorescence half-time of FRET-positive and FRET-negative cells. The significant difference between the two experiments indicates that PEX3-EYFP interacted with ECFP-PEX19.

**Fig. 6.** Quantification of FRET by donor photobleaching FRET microscopy. PEX3-deficient human fibroblasts were co-transfected with PEX3-EYFP/ECFP-PEX19 and with PEX3-EYFP/ECFP, respectively, and three days after transfection cells were processed for immunofluorescence analysis and bleached using the donor excitation wavelength of 458 nm (a). Fluorescence intensity was measured in the regions of interest and fitted according to the equation given under “Materials and methods” to obtain a donor fluorescence decay curve and the relative donor fluorescence half-time. The decay of fluorescence intensity was less pronounced when ECFP-PEX19 was co-transfected with PEX3-EYFP (▲, n = 22, FRET positive) than upon co-transfection of the empty vector ECFP with PEX3-EYFP (●, n = 17, FRET negative).

**Fig. 7.** Donor photobleaching FRET microscopy using PEX3 deletion constructs. PEX3-deficient human fibroblasts were transfected with plasmids encoding two different PEX3 deletion constructs each in combination with the cDNA for ECFP-PEX19 or ECFP, respectively. Results obtained using the ECFP-PEX19 partner were compared to those using ECFP. The boxes represent the 50 percent confidence interval (25th – 75th percentile); the horizontal black bars represent the medians; the error bars indicate the minimum-maximum range. The p values refer to the difference between the mean relative donor fluorescence half-time of cells transfected with the plasmids encoding the PEX3 deletion constructs and their individual control, respectively. The lack of significant difference to the control experiments indicates that PEX3 34–373-EYFP and PEX3 1–140-EYFP did not interact with ECFP-PEX19, respectively.
 different in vitro assays (Fransen et al., 2001; Mayerhofer et al., 2002).

We used a second PEX3 deletion construct, PEX3 1–140-EYFP, to investigate whether a lack of the PEX19-binding domain of PEX3, mapped to the C-terminal portion of the protein (Fransen et al., 2001), would disrupt the interaction with ECFP-PEX19. Donor fluorescence photobleaching experiments indeed revealed no significant difference of the relative fluorescence half-time when the interaction between PEX3 1–140-EYFP and ECFP-PEX19 was compared to the interaction between PEX3 1–140-EYFP and ECFP. Several other C-terminal truncations of PEX3 from different species have previously been shown by different techniques not to interact with PEX19 (Soukupova et al., 1999; Ghadeli et al., 2000; Muntau et al., 2000; Fransen et al., 2001), and our data confirm these findings.

In conclusion, our results highlight the potential of FRET microscopy in revealing quantitative spatial information about the physical interaction of peroxins during the process of peroxisome biogenesis in living cells. Thereby data from FRET analysis provide complementary information to those obtained by other approaches such as yeast two-hybrid screens and biochemical co-immunoprecipitation experiments. FRET also offers the prospect to be used for kinetic studies of protein interactions during organelle assembly. Our results indicate that the main site of interaction between PEX3 and PEX19 is the peroxisomal membrane and not the cytosolic compartment. These findings have implications for the current hypotheses concerning the functional role of PEX3 and PEX19 in that they are consistent with the hypothesis of PEX3 being an anchor for PEX19 at the peroxisomal membrane.

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