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Targeting of hFis1 to Peroxisomes Is Mediated by Pex19p*[S]

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The processes of peroxisome formation and proliferation are still a matter of debate. We have previously shown that peroxisomes share some components of their division machinery with mitochondria. hFis1, a tail-anchored membrane protein, regulates the membrane fission of both organelles by DLP1/Drp1 recruitment, but nothing is known about the mechanisms of the dual targeting of hFis1. Here we demonstrate for the first time that peroxisomal targeting of hFis1 depends on Pex19p, a peroxisomal membrane protein import factor. hFis1/Pex19p binding was demonstrated by expression and co-immunoprecipitation studies. Using mutated versions of hFis1 an essential binding region for Pex19p was located within the last 26 C-terminal amino acids of hFis1, which are required for proper targeting to both mitochondria and peroxisomes. The basic amino acids in the very C terminus are not essential for Pex19p binding and peroxisomal targeting, but are instead required for mitochondrial targeting. Silencing of Pex19p by small interference RNA reduced the targeting of hFis1 to peroxisomes, but not to mitochondria. In contrast, overexpression of Pex19p alone was not sufficient to shift the targeting of hFis1 to peroxisomes. Our findings indicate that targeting of hFis1 to peroxisomes and mitochondria are independent events and support a direct, Pex19p-dependent targeting of peroxisomal tail-anchored proteins.

In recent years it has become apparent that peroxisomes and mitochondria are two organelles having more in common than previously assumed (1). Besides their metabolic interplay, for example in fatty acid β-oxidation, lipid homeostasis, and reactive oxygen species metabolism, an overlap in the division machinery of both organelles was also discovered (2). The dynamin-like protein DLP1/Drp1 known to participate in mitochondrial fission (3, 4) was shown to be involved in peroxisomal division as well (5, 6), where it is required for the final scission of the organelles (3, 4, 7). A patient with a mutation in *DLP1* has recently been described (8). The DLP1 mutation results in a lethal disorder whereby fission of both mitochondria and peroxisomes is impaired. These findings further underline the importance of organelle dynamics for health and disease (9, 10).

Mitochondrial fission is regulated at least partly by hFis1, a 17-kDa tail-anchored (TA)[2] protein of the mitochondrial outer membrane (4, 11, 12). hFis1, as the yeast homolog Fis1p, possesses a single transmembrane domain located close to a short C terminus protruding in the lumen of the organelle (4, 13). The N-terminal cytosolic domain forms a tetratricopeptide-repeat-like helix bundle (14–16), which is supposed to interact with DLP1/Drp1 (17). We and others have recently demonstrated that hFis1 also localizes to the peroxisomal membrane and that it is likewise mediating peroxisomal fission by recruiting DLP1/Drp1 to peroxisomes (18, 19). Increasing the amount of hFis1 on peroxisomes and mitochondria has been shown to promote peroxisomal and mitochondrial division (4, 18). Apparently, both organelles have to compete for DLP1, and thus, hFis1 has a key function in recruiting DLP1 to peroxisomes and mitochondria. A function of yeast Fis1p in peroxisomal division has also been reported (20).

So far, hFis1 is one of the few membrane proteins known to be targeted to both peroxisomes and mitochondria (2). However, it is currently not understood how this dual targeting is achieved. Very recently, another tail-anchored protein, Mff, has been identified, which is targeted to both peroxisomes and mitochondria, and is supposed to play a role in organelle division/dynamics as well (21).

Due to the proximity of the transmembrane domain to the C terminus, TA proteins show a particular mode of biogenesis and have to be inserted into their target membranes post-translationally (for a recent review see Ref. 22). The targeting signals of TA proteins for the sorting to and insertion into the respective membranes are generally encoded in their C termini, including the transmembrane domain (22–24).

The import signal of mitochondrial TA proteins is in the main a transmembrane domain with moderate length and hydrophobicity, flanked by basic amino acids (22). Although the TOM complex mediates the import of most mitochondrial membrane proteins (25, 26), there is evidence that the targeting of mitochondrial TA proteins is independent of this complex (27, 28).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) NM_003847 and NM_002857.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[2] The abbreviations used are: TA, tail-anchored; ER, endoplasmic reticulum; GFP, green fluorescent protein; IP, immunoprecipitation; MDV, mitochondria-derived vesicle; Pex, peroxin; PMP, peroxisomal membrane protein; TRITC, tetramethylrhodamine isothiocyanate; YFP, yellow fluorescent protein; HA, hemagglutinin; siRNA, small interference RNA; DSP, dithiobis(succinimidylpropionate); MOPS, 4-morpholinepropanesulfonic acid.

Studies on TA protein targeting to peroxisomes are scarce, especially in mammalian cells (29–33), and have very recently been further complicated by the discovery of a novel vesicular trafficking pathway from mitochondria to peroxisomes involving mitochondria-derived-vesicles (MDVs) (34). Thus, delivery of membrane (and TA) proteins to peroxisomes *per se* can be mediated by direct insertion from the cytosol, by transit through the ER (or a subdomain) (35), and via mitochondria by a population of MDVs. However, most peroxisomal membrane proteins (PMPs) are likely to be inserted into peroxisomes directly from the cytosol. This process requires Pex19p, a mainly cytosolic protein that acts as chaperone and/or import factor for most PMPs (36–41) and directs them to the peroxisomal membrane by interaction with Pex3p (42–45). In a recent study, it has been reported that the peroxisomal TA proteins Pex26p and yeast Pex15p utilize the regular machinery for the import of PMPs and are targeted by binding of Pex19p (33).

In this study we have investigated the membrane targeting of mammalian hFis1. We show that the C-terminal domain of hFis1 contains a Pex19p binding site, and that Pex19p is required for peroxisomal targeting of hFis1. Pex19p binding is independent of basic amino acid residues in the C terminus of hFis1, which are instead required for mitochondrial targeting. We provide first evidence for a direct, Pex19p-dependent targeting of tail-anchored hFis1 to peroxisomes.

EXPERIMENTAL PROCEDURES

cDNAs and Antibodies—hFis1 fused to green fluorescent protein (GFP), or the Myc epitope tag (GFP-hFis1 and Myc-hFis1), the C-terminally truncated hFis1 constructs (Myc-hFis1-ΔC and Myc-hFis1-ΔTM/C), and a construct encoding the C-terminal 26 amino acids of hFis1 tagged to yellow fluorescent protein (YFP) (hFis1-YFP-TM/C) were described previously (4, 18). Constructs encoding hFis1 carrying point mutations and fused to GFP (GFP-hFis1^{K149A}, GFP-hFis1^{K151A}, and GFP-hFis1$^{K149/151A}$) were kindly provided by M. T. Ryan (La Trobe University, Melbourne, Australia (12)) (Fig. 1). Pex19 fused to YFP or the HA epitope tag (YFP-Pex19 and HA-Pex19) was a gift from P. U. Mayerhofer (Ludwig-Maximilians-University, Munich, Germany). Pex11pβ C-terminally fused to the Myc epitope tag (Pex11pβ-Myc) was described previously (46). Human cDNA was produced from mRNA isolated from HepG2 cells. The following primer sequences (MWG, Martiensried, Germany) were used to amplify the coding sequence of hsPex11pα (accession number NM_003847) from this cDNA by nested PCR: 5′-CCAGAGGACCCACGCCTGAGCC-3′ (outer forward primer), 5′-ATGTCTGTCCCACCAAGAGGCC-3′ (outer reverse primer), 5′-TTGAATTCATGGACGCCTTCACCCGCTTC-3′ (inner forward primer) and 5′-GAAGATCTCGGGTCTTCAGCTTCATCG-3′ (inner reverse primer). Using the restriction sites for EcoRI and BglII at the ends of the final PCR product (underlined) Pex11α cDNA was inserted in-frame into the pEYFP-N1 vector (Clontech, Saint-Germain-en-Laye, France) using EcoRI and BamHI and verified by sequencing (MWG). Rabbit anti-PMP70 antibody was kindly provided by A. Völkl (University of Heidelberg, Germany), and rabbit anti-hTom22C antibody was a gift from M. T. Ryan (La Trobe University). Rabbit anti-hFis1 (a kind gift from Y. Yoon, University of Rochester) and anti-GFP (Invitrogen; recognizes also YFP) antibodies were used for immunoblotting, as well as mouse anti-Pex19p antibody (BD Biosciences, San Jose, CA) and mouse anti-VDAC1 antibody (Abcam, Cambridge, UK). Monoclonal anti-GFP antibody (Clontech; recognizes also YFP) and rabbit anti-Fis1 (Alexis Biochemicals, Lausen, Switzerland) were used for immunoprecipitation studies, monoclonal anti-HA epitope antibody (Covance, Princeton, NJ), anti-Myc epitope 9E10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Tom20 antibody (BD Biosciences Pharmingen, San Diego, CA) were used for immunoblotting and immunofluorescence. Specific anti-IgG antibodies conjugated to tetramethylrhodamine 5-isothiocyanate (TRITC) or to Alexa488 were obtained from Dianova (Hamburg, Germany), Invitrogen, and MoBiTec (Göttingen, Germany).

Cell Culture, RNA Interference, and Transfection Experiments—COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum as described previously (47). For morphological studies the cells were transfected with DNA constructs by incubation with polyethylenimine (Sigma-Aldrich) or via electroporation (46, 48). For immunoprecipitation studies the cells were transfected with DEAE-dextran (Amersham Biosciences/GE Healthcare, Munich, Germany). To knock down the expression of Pex19p (accession number NM_002857) by RNA interference, 21-nucleotide small interfering RNA (siRNA) was transfected into the cells by electroporation (49). The Pex19-specific siRNA oligonucleotides were obtained as pre-designed siRNAs from Ambion (Austin, TX) (5′-GGAGAUCACAGAAAAGUAUtt-3′, 5′-GGAGACACUGCCAAAGAUGtt-3′, and 5′-GGAACUAUUCGACAGUGAAtt-3′) and adopted from Jones *et al.* (39) (5′-GAGAUCGCCAGGAGACACUtt-3′). As controls, cells were either transfected with buffer or with non-targeting siRNA. 48 h after transfection with siRNA the cells were transfected by electroporation with DNA encoding for GFP-hFis1 or Pex11pβ-Myc and morphologically assayed for peroxisomal import after 3, 6, and 24 h.

Immunofluorescence and Microscopy—Cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, permeabilized with 0.2% Triton X-100 or 25 μg/ml digitonin and incubated with primary and secondary antibodies as described (46). Transfected cells were processed for immunofluorescence 3, 6, 24, or 48 h after transfection. Samples were examined using an Olympus IX81 microscope (Olympus Optical, Hamburg, Germany) equipped with a PlanApo 100×/1.40 oil objective. Fluorescence images were acquired with an F-view II CCD camera (Soft Imaging System GmbH, Münster, Germany) driven by Soft imaging software. Confocal images were acquired on an LSM-510 confocal microscope (Carl Zeiss, Oberkochen, Germany) using a ×100 objective. Digital images were optimized for contrast and brightness using appropriate software. For quantitative analysis of peroxisomal morphology, 100–200 cells per coverslip were examined and categorized as cells with spherical (0.1–0.3 μm) or tubular (2–5 μm in length) peroxisomes as described previously (50). Fluorescence intensities were determined on digital images by encircling single peroxisomes (or mitochondria), and

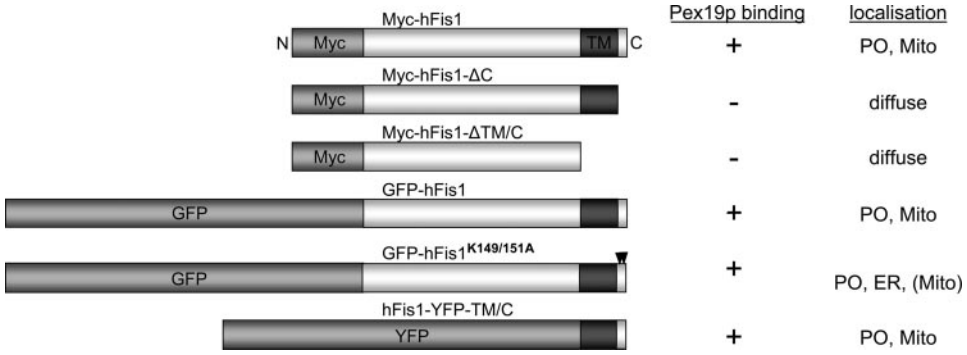

FIGURE 1. **Overview of the hFis1 constructs used in this study.** The *bars* on the *left side* represent the proportion of the protein domains of the different tagged and/or truncated hFis1 constructs. Myc-hFis1 and GFP-hFis1 are full-length hFis1 constructs N-terminally fused with a Myc epitope tag or GFP, respectively. Myc-hFis1-ΔC has a C-terminal truncation of 5 amino acids whereas the Myc-hFis1-ΔTM/C construct lacks 26 amino acids (the transmembrane domain (*TM*) and the C terminus). In hFis1-YFP-TM/C these 26 amino acids are fused to the C terminus of YFP. GFP-hFis1$^{K149/151A}$ is a full-length construct carrying lysine to alanine mutations in the residues 149 and 151 (*arrowheads*). An overview of the particular binding to Pex19p and the subcellular localization is given on the *right*. *ER*, endoplasmic reticulum; *Mito*, mitochondria; and *PO*, peroxisomes.

the GFP/TRITC ratio was calculated. Images were processed and quantified using LSM-510 software (Carl Zeiss MicroImaging, Inc.). Usually two coverslips per preparation were analyzed and three to five independent experiments were performed. Significant differences between experimental groups were detected by analysis of variance for unpaired variables using Microsoft Excel software. Data are presented as means ± S.D., with an unpaired *t* test used to determine statistical differences. p values <0.05 were considered as significant, and p values <0.01 were considered as highly significant.

Immunoprecipitations—To study the interaction of Pex19p and hFis1, COS-7 cells were transfected with different hFis1 and Pex19p constructs or not transfected for detection of endogenous interactions. To stabilize potentially transient or weak interactions, in some experiments whole cells were subjected to chemical cross-linking by incubation with the cleavable cross-linker dithiobis(succinimidylpropionate) (DSP, Pierce) as described (4, 18). Cells were lysed after 48 h by adding lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Triton-X-100) containing a protease inhibitor mixture, passed through a 26.5-gauge syringe needle ten times and incubated by mixing at 4 °C for 30 min. Lysates were cleared by centrifugation (15,000 × *g* for 15 min). In case of immunoprecipitation (IP) with anti-GFP/YFP or anti-Fis1 antibodies, a preclearing with 30 μl of Protein-A-Sepharose (Sigma-Aldrich) was performed. The cleared lysates were afterward preincubated with the respective antibodies for 1 h. IP was performed by adding 50 μl of Protein-A-Sepharose or 25 μl of a 25% slurry of anti-Myc 9E10 monoclonal antibodies conjugated to agarose beads (Santa Cruz Biotechnology) and incubation with mixing at 4 °C overnight. The beads were extensively washed with wash buffer 1 (1× phosphate-buffered saline, 0.5% Triton X-100, 0.5% sodium deoxycholate), followed by wash buffer 2 (500 mM NaCl, 125 mM Tris/HCl, pH 8.0, 10 mM EDTA, 0.5% Triton-X-100), one time with phosphate-buffered saline and boiled in SDS-PAGE sample buffer, which also cleaves the cross-linker DSP. The immunoprecipitated proteins and the corresponding input (load) were separated on 12.5% polyacrylamide gels by SDS-PAGE and analyzed by immunoblotting. To study the association of endogenous hFis1 with peroxisomes, peroxisomal membranes were immunoprecipitated according to a previous study (6). COS-7 cells expressing Pex11pα-YFP were homogenized in homogenization buffer (5 mM MOPS, pH 7.4, 250 mM sucrose, 1 mM EDTA, protease inhibitor mixture) by passing gently through a 26.5-gauge syringe needle. The homogenate was cleared by centrifugation (500 × *g* for 5 min), and peroxisomes were subfractionated by centrifugation at 25,000 × *g* for 25 min. The organelle pellet was then gently resuspended in hypotonic lysis buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA), and the peroxisomal membranes were immunoprecipitated by preincubation for 1 h with anti-GFP/YFP antibodies, followed by overnight incubation with Protein-A-Sepharose. The beads were extensively washed with TBS buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) (6), and the samples were analyzed by SDS-PAGE and immunoblotting using anti-hFis1 antibody.

Gel Electrophoresis and Immunoblotting—Protein samples were separated by SDS-PAGE on 12.5% polyacrylamide gels, transferred to nitrocellulose using a semidry apparatus, and analyzed by immunoblotting. Immunoblots were processed using specific primary antibodies, horseradish peroxidase-conjugated secondary antibodies (Bio-Rad), and enhanced chemiluminescence reagents (GE Healthcare, Munich, Germany). For quantification, immunoblots were scanned with a Bio-Rad GS-710 calibrated imaging densitometer and processed using Bio-Rad Laboratories Quantity One software.

RESULTS

Interaction of Pex19p and hFis1—Pex19p is a chaperone involved in the targeting of peroxisomal membrane proteins (39, 51, 52). Recently, it was shown that Pex19p functions as a targeting factor for the peroxisomal TA protein Pex26p (33). To examine if Pex19p is also involved in peroxisomal targeting of hFis1, we first performed co-immunoprecipitation studies.

COS-7 cells were co-transfected with Myc-hFis1 and YFP-Pex19p constructs (Figs. 1, 2, and 3). After 48 h cells were lysed, and immunoprecipitations with anti-Myc as well as anti-GFP/YFP antibodies were performed. Myc-hFis1 was observed to co-precipitate with YFP-Pex19p (Fig. 2*A*, *upper panel*, *left*). Furthermore, YFP-Pex19p was found to co-precipitate with Myc-hFis1 (Fig. 3*A*, *lane a*). These results strongly suggest that Pex19p and hFis1 are part of the same complex.

Interestingly, immunoprecipitations of Myc-hFis1 via YFP-Pex19p required the use of a cross-linker (DSP). For all other immunoprecipitations (*e.g.* with GFP-hFis1 or Pex11pβ-Myc), cross-linking could be omitted, and DSP was no longer used. As a positive control for our assay, we verified that Pex11pβ-Myc, which was previously shown to bind Pex19p (39, 40), was able to

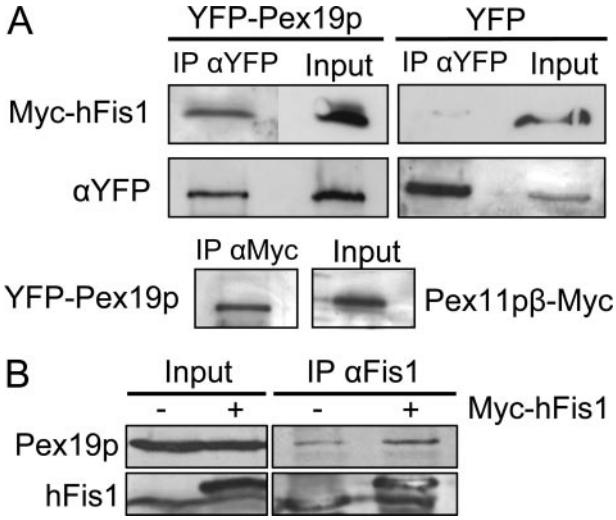

FIGURE 2. **Pex19p and hFis1 interact with each other.** *A*, COS-7 cells were co-transfected with Myc-hFis1 and YFP-Pex19p (on the *left*) or cytosolic YFP as negative control (pEYFP-N1; on the *right*), and incubated for 48 h. Whole cells were subjected to chemical cross-linking by adding DSP for 45 min. Immunoprecipitations (IPs) were performed with anti-GFP/YFP antibodies and Protein A-Sepharose followed by analysis of the samples by SDS-PAGE and immunoblotting using anti-Myc and anti-GFP/YFP antibodies. Note that Myc-hFis1 co-precipitated with YFP-Pex19p, but not with cytosolic YFP. The *lower panel* shows a positive control experiment without DSP. Pex11pβ-Myc, which is known to interact with Pex19p, forms a complex with YFP-Pex19p. *B*, COS-7 cells were transfected with Myc-hFis1 or not transfected. Immunoprecipitation of endogenous (and overexpressed) hFis1 was performed using anti-Fis1 antibodies and Protein A-Sepharose, and the co-precipitation of endogenous Pex19p was detected by immunoblotting using anti-Pex19 antibodies.

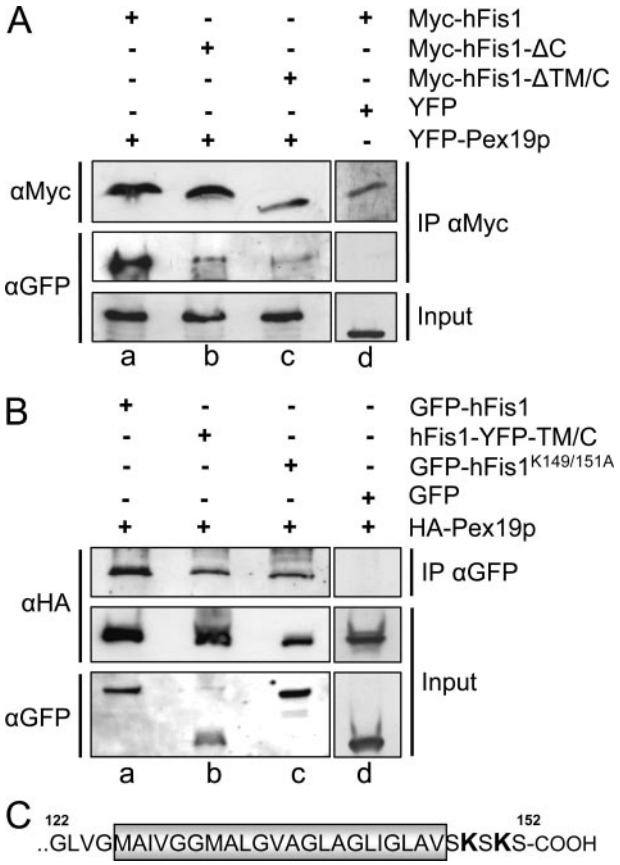

FIGURE 3. **The Pex19p binding region is located in the C terminus of hFis1 and does not require basic amino acids.** *A*, co-transfection of COS-7 cells with YFP-Pex19p and Myc-hFis1 (*lane a*), Myc-hFis1-ΔC (*lane b*), or Myc-hFis1-ΔTM/C (*lane c*) followed by immunoprecipitation (IP) with anti-Myc antibodies conjugated to agarose beads. The analysis of the samples was performed by immunoblotting using anti-GFP/YFP antibodies. Note that Pex19p binding to hFis1 is impaired after removal of the C-terminal tail or the transmembrane domain of hFis1 (*lanes b* and *c*). To exclude unspecific co-precipitations control experiments were performed with cytosolic YFP (pEYFP-N1) and Myc-hFis1 (*lane d*). *B*, COS-7 cells were co-transfected with HA-Pex19p and GFP-hFis1 (*lane a*), hFis1-YFP-TM/C (*lane b*), GFP-hFis1$^{K149/151A}$ (*lane c*), or cytosolic GFP as negative control (pEGFP-N1; *lane d*). IPs were performed by adding anti-GFP/YFP antibodies and Protein A-Sepharose. The samples were analyzed by immunoblotting with anti-HA antibodies. Note that the C-terminal domain of hFis1 (*TM/C*, 26 amino acids) fused to YFP is sufficient to co-precipitate HA-Pex19p (*lane b*) and that mutations of the basic amino acids in the very C terminus of hFis1 do not abolish binding to HA-Pex19p (*lane c*). *C*, amino acid sequence of the C terminus (amino acids 122–152) of hFis1. The transmembrane domain is *boxed*, and basic amino acids (Lys-149 and Lys-151) are in *bold*. Quantitative analyses of *A* and *B* are shown in supplemental Fig. S1.

immunoprecipitate YFP-Pex19p, also without usage of DSP (Fig. 2*A*, *lower panel*).

Furthermore, the interaction of endogenous Pex19p with endogenous or expressed hFis1 was studied. COS-7 cells not transfected or transfected with only Myc-hFis1 were subjected to immunoprecipitations with anti-Fis1 antibodies. Endogenous Pex19p was found to co-precipitate with endogenous hFis1, and the amount of co-precipitated Pex19p increased when additionally Myc-hFis1 was expressed (Fig. 2*B*). These data further support an interaction between hFis1 and Pex19p.

Pex19p Binds to the C Terminus of hFis1—We and others have recently shown that an intact C terminus of hFis1 is necessary and sufficient for peroxisomal and mitochondrial targeting (4, 18). Removal of the short C-terminal tail consisting of five amino acids impaired proper targeting of hFis1 to peroxisomes and mitochondria. To further characterize the interaction of Pex19p and hFis1, different deletion mutants of hFis1 were used for co-immunoprecipitations (Figs. 1 and 3). The mutant Myc-hFis1-ΔC lacks the 5 C-terminal amino acids (C-tail), whereas Myc-hFis1-ΔTM/C misses the last 26 amino acids, which contain the C-terminal tail and the transmembrane domain. Recent morphological studies revealed that both truncated proteins are targeted neither to peroxisomes nor to mitochondria; instead they showed a more diffuse, cytoplasmic localization (4, 18). In line with the morphological observations, YFP-Pex19p was reduced or nearly absent from immune complexes obtained by precipitation of Myc-hFis1-ΔC or Myc-hFis1-ΔTM/C in contrast to wild-type Myc-hFis1 (Fig. 3*A* and supplemental Fig. S1 for a quantitative analysis). Noteworthy, the removal of only five amino acids from the C terminus of hFis1 impedes the binding of Pex19p.

To confirm the assumption that Pex19p binds to the C terminus of hFis1, hFis1-YFP-TM/C was used for co-immunoprecipitations. This construct consists of the last 26 amino acids of hFis1 (the transmembrane domain and the C-tail) fused to the C terminus of YFP (Fig. 1). It was previously shown that this construct is properly targeted to peroxisomes and mitochondria (18). Co-expression of HA-Pex19p and GFP-hFis1 (control) or hFis1-YFP-TM/C, followed by immunoprecipitations using anti-GFP/YFP antibodies, revealed a co-precipitation of HA-Pex19p with both proteins (Fig. 3*B*). Thus, it appears that the C-terminal tail of hFis1 together with the transmembrane

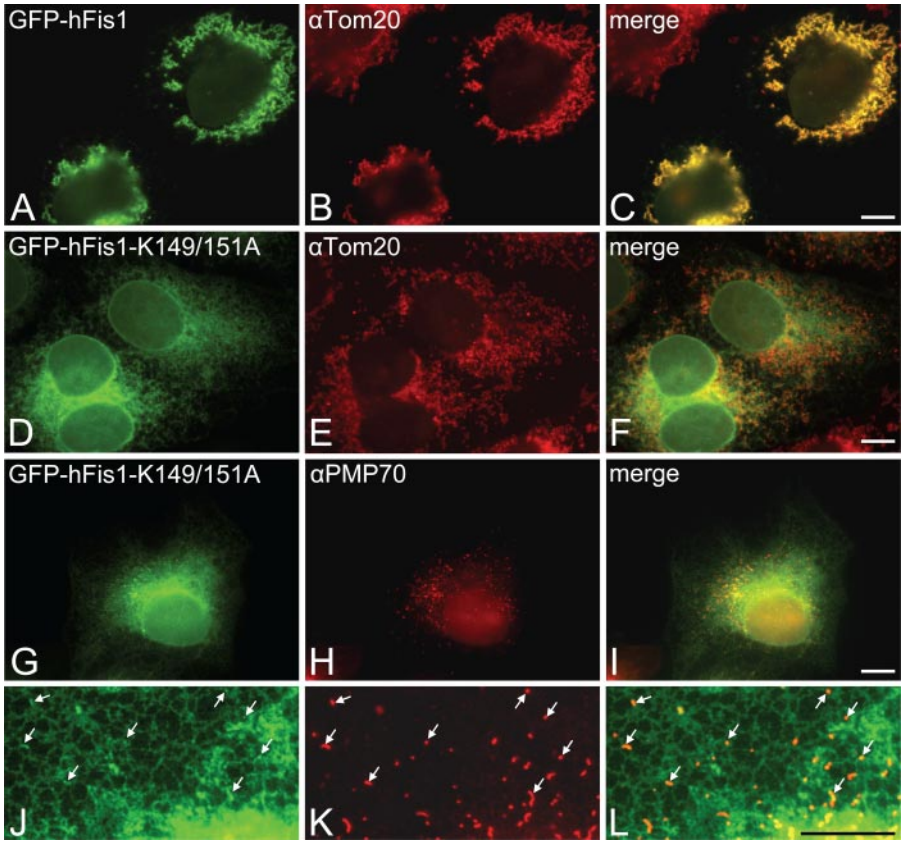

FIGURE 4. **C-terminal basic amino acids are not required for peroxisomal targeting of hFis1.** COS-7 cells expressing GFP-hFis1$^{K149/151A}$ (*D–L*) or GFP-hFis1 (*A–C*) were processed for immunofluorescence using anti-PMP70 antibodies to label peroxisomes (*H* and *K*) or anti-Tom20 antibodies to label mitochondria (*B* and *E*). *G–I*, overview of a representative cell. *J–L*, higher magnification view. *Arrows* highlight some regions of colocalization. Overlays (*merge*) are shown on the *right*. Note that GFP-hFis1$^{K149/151A}$ is still prominently targeted to peroxisomes, whereas staining of mitochondria is weak. In addition, GFP-hFis1$^{K149/151A}$ is misdirected to the tubulo-reticular network of the ER. *Bars*, 10 μm. See also supplemental Fig. S2 for a peroxisomal co-labeling of cells expressing GFP-hFis1.

domain is sufficient and necessary to bind Pex19p and to target hFis1 to peroxisomes.

Mutated hFis1$^{K149/151A}$ Localizes to Peroxisomes and Binds Pex19p—Basic amino acids close to a transmembrane domain were shown to be important for targeting of C-tail anchored proteins (22) as well as for general targeting of peroxisomal membrane proteins (35). The basic amino acids in the C-terminal tail of hFis1 (Fig. 3*C*) are supposed to be part of a targeting signal. hFis1 contains two lysine residues at positions 149 and 151, and this overall basic charge was shown to be important for proper mitochondrial targeting of hFis1 (12). To examine whether the targeting to peroxisomes also depends on these lysine residues, we studied the targeting and Pex19p binding of hFis1 carrying point mutations K149A and/or K151A. In these mutants either of the lysine residues alone or both had been mutated to alanine. As reported recently, unlike for GFP-hFis1 (Fig. 4, *A–C*, and supplemental Fig. S2), the mutant GFP-hFis1$^{K149/151A}$ was not properly targeted to mitochondria, and some misdirection to the ER was observed (Fig. 4, *D–F*) (12). When GFP-hFis1$^{K149/151A}$ was expressed in COS-7 cells, it still localized to peroxisomes (Fig. 4, *G–L*). The majority of the peroxisomes in a given cell showed co-localization of GFP-hFis1$^{K149/151A}$ with the peroxisomal marker PMP70, whereas mitochondrial localization of GFP-hFis1$^{K149/151A}$ was weak (Fig. 4). However, misdirection of GFP-hFis1$^{K149/151A}$ to the ER was also observed probably due to a disturbed mitochondrial import (12). In addition, the single mutants GFP-hFis1^{K151A} and GFP-hFis1^{K149A} were properly targeted to peroxisomes (data not shown). We conclude that the overall basic charge within the C-terminal tail of hFis1 is not crucial for peroxisomal targeting.

Next we investigated if the two C-terminal lysine residues of hFis1 are required for the binding of Pex19p to hFis1. Co-immunoprecipitations of HA-Pex19p with GFP-hFis1$^{K149/151A}$ revealed to be just as effective as with wild type GFP-hFis1 (Fig. 3*B* and supplemental Fig. S1 for a quantitative analysis). These findings confirm the morphological data obtained (Fig. 4), and indicate that the overall basic charge within the C-terminal tail of hFis1 is not essential for binding of Pex19p. For an overview of the hFis1 constructs used, their Pex19p-binding properties and their subcellular localizations see Fig. 1.

Targeting of hFis1 to Peroxisomes Is Not Increased after Overexpression of Pex19p—Next, we examined if overexpression of Pex19p leads to a shift in the targeting of hFis1 toward peroxisomes. To specifically label peroxisomal membranes in COS-7 cells, we expressed a Pex11pα-YFP construct. Pex11pα-YFP, which exposes its N and C termini toward the cytosol (53), localized specifically to peroxisomes (Fig. 5, *A–E*). In contrast to other peroxisomal membrane proteins (for example, Pex11pβ) (46), no morphological alterations of the peroxisomal compartment were induced by Pex11pα-YFP, which might have influenced the association of hFis1 (Fig. 5, *A–E*).

COS-7 cells were transfected with Pex11pα-YFP alone or co-transfected with HA-Pex19p. After careful homogenization of the cells, peroxisomes were enriched by subcellular fractionation. The resulting organelle pellet was gently resuspended in a hypotonic lysis buffer to generate membranes, and peroxisomal membranes were specifically precipitated by anti-GFP/YFP antibodies (6). The relative amounts of membrane-associated, endogenous hFis1 were quantified after immunoblotting with anti-hFis1 antibodies. Only a very slight increase in the amount of hFis1 associated with peroxisomal membranes after overexpression of Pex19p was observed. Data were normalized using Pex11pα as input references (Fig. 5, *F* and *G*). Levels of mitochondrial marker proteins (for example, VDAC1 and Tom20) were found to be decreased in peroxisome-enriched fractions,

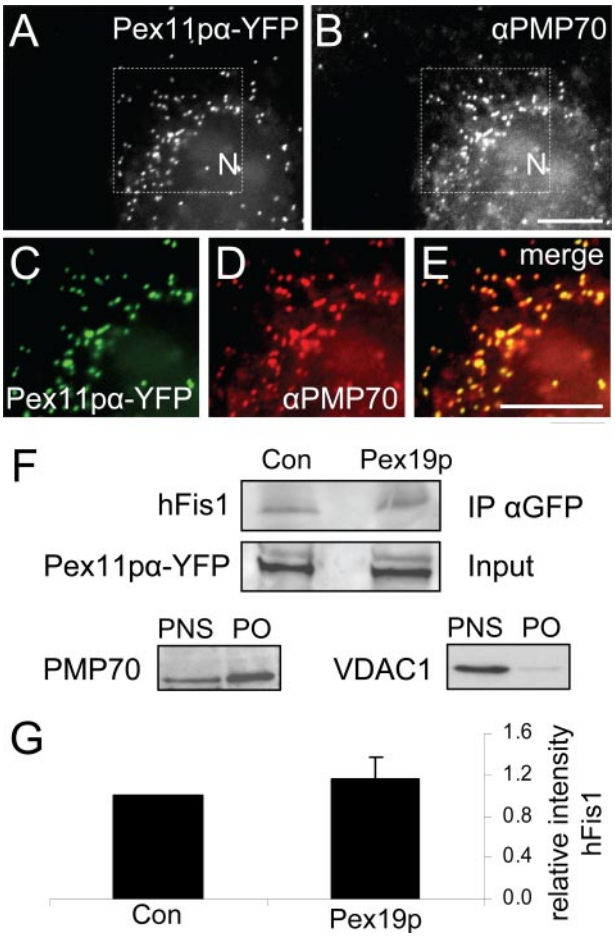

FIGURE 5. **Overexpression of Pex19p does not increase peroxisomal targeting of hFis1.** *A–E*, Pex11pα-YFP is properly targeted to peroxisomes. COS-7 cells expressing Pex11pα-YFP were immunostained with an antibody to PMP70 (*B* and *D*). *C–E*, higher magnification view of the *boxed regions* in *A* and *B*. *E*, overlay (*merge*) of *C* and *D*. *F*, peroxisome immunoprecipitation. COS-7 cells were transfected with Pex11pα-YFP alone (*Con*) or co-transfected with HA-Pex19p. Peroxisomal membranes were immunoprecipitated from peroxisome-enriched fractions using anti-GFP/YFP antibodies and Protein A-Sepharose. Endogenous hFis1 was detected by immunoblotting with anti-hFis1 antibodies. Contamination of mitochondria in the peroxisomal membrane fractions (PO) was low, as shown by immunoblotting with VDAC1 antibodies in the *lower panel*, in contrast to the enrichment of peroxisomal markers (PMP70). Equal amounts of protein (30 μg/lane) were loaded onto the gels. *G*, quantitative analysis of the anti-hFis1 immunoblots. Data were normalized using Pex11pα as input references. The data are from four independent experiments and are expressed as means ± S.D. *N*, nucleus. *PNS*, postnuclear supernatant. *Bars*, 10 μm.

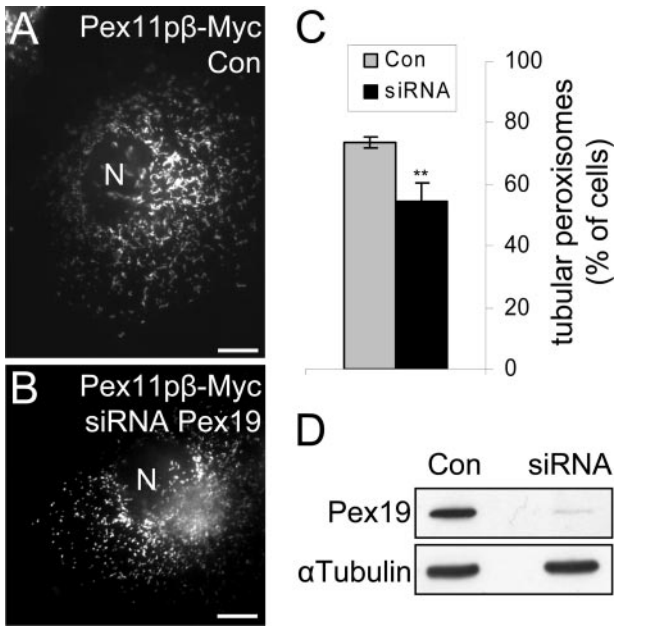

FIGURE 6. **Silencing of Pex19p reduces peroxisomal proliferation induced by Pex11pβ.** COS-7 cells were transfected with Pex19p siRNA duplexes. After 48 h the cells were transfected with Pex11pβ-Myc and after additional 6 h processed for immunofluorescence using anti-Myc (and anti-PMP70) antibodies. *A*, control cell (*Con*) with typical tubular, elongated peroxisomes induced by Pex11pβ expression. *B*, silencing of Pex19p (*siRNA*). In many cases, the peroxisomes exhibit a spherical morphology and are not elongated. *C*, quantitative evaluation of peroxisome morphology. Note the reduced frequency of tubular peroxisomes in cells silenced for Pex19p. The data are from four independent experiments and are expressed as means ± S.D. (**, $p < 0.01$). *D*, immunoblots of cell lysates prepared after 48 h from control (*Con*) and silenced cells (*siRNA*) using anti-Pex19p and anti-tubulin antibodies (loading control). Equal amounts of protein (35 μg/lane) were loaded onto the gels. *N*, nucleus. *Bars*, 10 μm.

and they were absent or only present in very small, but equal amounts in immunoprecipitations (Fig. 5*F* and not shown). Because a prominent shift of the targeting of hFis1 toward peroxisomes was not induced by solely overexpressing Pex19p, other rate-limiting factors of the peroxisomal import machinery (for example, Pex3p) are likely to be involved.

Peroxisomal Proliferation Induced by Pex11pβ Depends on Pex19p—To further investigate the role of Pex19p in the peroxisomal targeting of hFis1, RNA interference experiments were performed. It was previously shown that the knock down of Pex19p with siRNA reduces the import of PMP34 (39) into peroxisomes. Furthermore, silencing of Pex19p leads to mistargeting of Pex26p to mitochondria (33). Transfection of COS-7 cells with Pex19 siRNA duplexes resulted in a prominent decrease in the protein level of Pex19p (Fig. 6*D*). As also observed by others (33, 39), peroxisomes were still present, even after extended knock down, and the levels of peroxisomal membrane proteins (PMP70, Fig. 7, and Pex14p (33)) were not grossly affected.

To prove the functionality of the Pex19p knockdown, its effect on peroxisome morphology after expression of Pex11pβ was assayed. The expression of Pex11pβ in mammalian cells induces a massive proliferation of peroxisomes, which includes elongation (growth) of the peroxisomal compartment followed by division into small spherical peroxisomes (46, 54). Especially the elongation step is easy to detect and to quantify by microscopic analysis. In previous studies it was shown that yeast Pex11p and human Pex11pβ bind to Pex19p (39, 40) (Fig. 2*A*). We investigated whether the knockdown of Pex19p would have an effect on the insertion of Pex11pβ into peroxisomal membranes and therefore on peroxisome elongation. Two days after silencing of Pex19p, COS-7 cells were transfected with Pex11pβ-Myc by electroporation and processed for immunofluorescence after 6–12 h. After silencing of Pex19p, the elongation of peroxisomes in Pex11pβ-Myc-expressing cells was significantly reduced (55 ± 5.58%, Fig. 6, *B* and *C*) compared with control cells (74 ± 1.71%, Fig. 6, *A* and *C*). Furthermore, many cells with a diffuse or granular, cytoplasmic distribution of Pex11pβ-Myc were observed (not shown). These findings indicate that silencing of Pex19p is functional in COS-7 cells. It

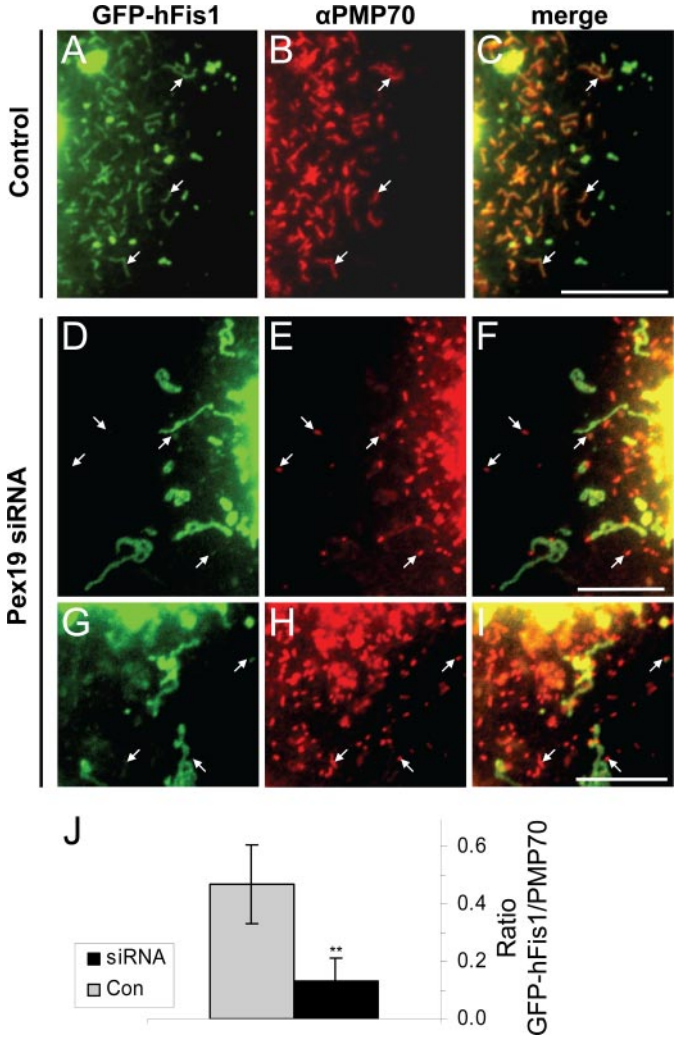

FIGURE 7. **Silencing of Pex19p inhibits the peroxisomal targeting of hFis1.** Pex19p in COS-7 cells was silenced by transfection with siRNA duplexes (*D–I*). After 48 h the cells were transfected with GFP-hFis1, and the targeting of GFP-hFis1 to peroxisomes was assayed after 3 h. Peroxisomes were immunostained with antibodies to PMP70 (*B*, *E*, and *H*). Co-localization of GFP-hFis1 and peroxisomes in control cells (*A–C*), and in cells silenced for Pex19p (*D–I*). Overlays (*merge*) are shown on the *right*. *Arrows* in (*A–C*) highlight regions of co-localization. Note the absence or reduction of co-localization (*arrows*) of GFP-hFis1 with PMP70 after silencing of Pex19p (*D–I*). *J*, fluorescence intensities of GFP-hFis and PMP-TRITC were determined on digital images by highlighting (*encircling*) single peroxisomes, and the GFP/TRITC ratios were calculated. 60–140 peroxisomes per cell were analyzed in ~60 control as well as treated cells. Data are expressed as means ± S.D. (**, $p < 0.01$) and are from three independent experiments. *N*, nucleus. *Bars*, 10 μm.

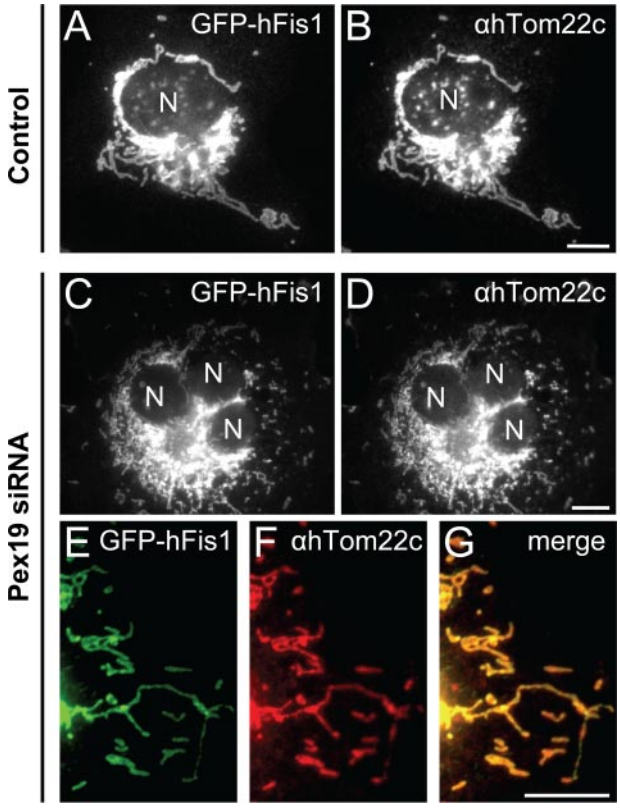

FIGURE 8. **Silencing of Pex19p does not inhibit mitochondrial targeting of hFis1.** COS-7 cells were processed as described in Fig. 7. Mitochondria were immunostained with an antibody to hTom22c, a mitochondrial outer membrane protein. *A* and *B*, control cells. *C–G*, COS-7 cells transfected with siRNA specific for Pex19p. *E–G*, higher magnification view. *G*, overlay (*merge*) of *E* and *F*. *N*, nucleus. *Bars*, 10 μm.

does not completely abolish but reduces the targeting of Pex11pβ to peroxisomes.

Targeting of hFis1 to Peroxisomes but Not to Mitochondria Depends on Pex19p—We now examined the effect of Pex19p knockdown on targeting of hFis1 to peroxisomes. Two days after silencing of Pex19p COS-7 cells were transfected with GFP-hFis1 by electroporation. The cells were prepared for immunofluorescence 3–5 h after transfection of GFP-hFis1 and examined for import of GFP-hFis1 into peroxisomes by co-localization with PMP70. In contrast to controls, the number of GFP-hFis1-positive structures that co-localized with peroxisomes labeled by PMP70 was decreased. Many PMP70-positive peroxisomes exhibited only a very weak staining for GFP-hFis1, or were not labeled at all (Fig. 7, *D–I*). In controls, the majority of the PMP70-positive peroxisomes showed co-labeling for GFP-hFis1 (Fig. 7, *A–C*) (18). These differences were less obvious after increased time points, likely due to the fact that silencing of Pex19p does not completely abolish import of PMPs (see above). In a quantitative approach, the fluorescence intensity of peroxisomal GFP-hFis1 and PMP70-TRITC was determined and the ratio calculated (Fig. 7*J*). In agreement with the morphological observations, the GFP-Fis1/PMP70-TRITC ratio was significantly reduced after silencing of Pex19p when compared with controls (Fig. 7). These data demonstrate that targeting of hFis1 to peroxisomes requires Pex19p.

A mistargeting of hFis1 to other organelles (for instance to the ER) as seen before for Pex26p and PMP34 (33, 39) did not occur. On the other hand, the knockdown of Pex19p did not influence the mitochondrial targeting of hFis1. Like in controls, GFP-hFis1 co-localized with the mitochondrial marker Tom22c, and mitochondria were observed to partially cluster around the nucleus as a result of GFP-hFis1 expression (Fig. 8, *A–D*, Fig. 4, *A–C*, and supplemental Fig. S2). A significant reduction or increase of hFis1 targeting to mitochondria after Pex19p knockdown was not detected by immunofluorescence (Fig. 8) or by ratio measurement of fluorescence intensities (not shown). The reduction of the peroxisomal import of hFis1 after silencing of Pex19p further supports the assumption that Pex19p functions as a cytosolic receptor for hFis1 targeting to peroxisomes.

DISCUSSION

Our study on the dual targeting of the TA protein hFis1 to peroxisomes and mitochondria provides firm evidence that peroxisome-targeted hFis1 uses the Pex19p-mediated targeting pathway. Our findings further support the recent assumption that in contrast to ER- and mitochondrial outer membrane-localized TA proteins, peroxisomal TA proteins make use of a protein-based sorting machinery, which is shared by other peroxisomal membrane proteins (22, 33). In a recent study, recognition sequences for Pex19p have been identified in the C-terminal regions of the peroxisomal TA proteins Pex26p and yeast Pex15p (33). The mPTS of hFis1 comprises its C-terminal 26 amino acids containing the transmembrane domain and the short 5-amino acid tail. This is consistent with the observation that targeting signals for TA proteins are generally located at the C termini and include the single transmembrane domain. Like most TA proteins, the luminal tail of hFis1 is very short (in contrast, that of Pex26p comprises 36 amino acids (33)). The C-terminal 26-amino acids of hFis1 are required for both peroxisomal and mitochondrial targeting, and are sufficient to direct an YFP fusion protein to both organelles (18). We could now show that the C-terminal 26 amino acids of hFis1 have Pex19p-binding properties. Removal of the C-terminal 26 amino acids or the 5-amino acid tail drastically diminished or abolished Pex19p binding as well as peroxisomal (and mitochondrial) targeting. In addition, a YFP fusion protein containing the C-terminal 26 amino acids was able to interact with Pex19p. These data demonstrate that the C terminus of hFis1 is sufficient and necessary for Pex19p binding and targeting to peroxisomes. In agreement with our experimental findings, a potential Pex19p binding site in the transmembrane region of hFis1 (amino acids 136–145) was also predicted by a recently developed prediction program (40, 55). In addition, two other potential binding sites have been predicted in the cytosolic, N-terminal part of hFis1. However, deletion of the transmembrane domain or the last C-terminal 5 amino acids of hFis1 drastically reduced the interaction with Pex19p. This indicates that the functional Pex19p binding site within the transmembrane domain is the essential one. In case the other binding sites are capable of binding Pex19p, they could allow weak interactions and assist in targeting and/or folding.

A role for Pex19p in peroxisomal targeting of hFis1 is further supported by siRNA experiments. Silencing of Pex19p was shown to significantly reduce the targeting of hFis1 to peroxisomes, but not to mitochondria. The import signal of mitochondrial TA proteins is supposed to be a transmembrane domain with moderate length and hydrophobicity, flanked by basic amino acids (22). hFis1 contains two lysine residues at positions 149 and 151, which are required for proper mitochondrial targeting (12). Interestingly, mutating either of the lysine residues alone or both to alanine, did not inhibit peroxisomal targeting of hFis1. Consistent with this, binding to Pex19p was also not abolished. These findings indicate that the overall basic charge within the C-terminal tail of hFis1 is not essential for binding of Pex19p and targeting to peroxisomes. In contrast, both positively charged lysine residues are important for mitochondrial targeting.

In contrast to peroxisomal TA proteins, which appear to utilize the regular machinery for the import of peroxisomal membrane proteins, TA protein import into the mitochondrial outer membrane was recently shown to be independent of the general import machinery, the TOM complex, and also from cytosolic factors (27, 28). So far, it is not clear if such an unassisted insertion applies to all mitochondrial TA proteins. Interestingly, native TA proteins were observed to compete with each other for delivery to the mitochondrial surface, thus implying the involvement of a *limiting factor* in folding or tail-mediated targeting (27). Furthermore, a role of the unique lipid composition of the mitochondrial outer membrane in the insertion of Fis1 has been recently proposed (28). hFis1 is supposed to be a key player in the regulation of peroxisomal as well as mitochondrial fission by recruitment of DLP1/Drp1 (4, 18). Organelle dynamics, such as fusion and fission events, are important for organelle function, and malfunction of the components involved has been related to several disease conditions (8–10). An increase in the amount of hFis1 at the organelle membranes has been shown to promote mitochondrial fragmentation as well as peroxisomal division (4, 18). Furthermore, both organelles might have to compete for recruitment of hFis1 under conditions of peroxisome proliferation and/or mitochondrial multiplication. It is therefore very likely that hFis1 recruitment to both peroxisomes and mitochondria and/or its function at the organelle membranes is regulated and controlled.

So far, Pex19p appears to directly recruit hFis1 out of the newly synthesized cytosolic pool to peroxisomes and might increase the specificity of this process. The remaining major pool of hFis1 is either inserted directly and without assistance into the mitochondrial membrane (28), or requires the help of a yet unknown chaperone (or import factor). This is similar to Pex26p, which is mainly targeted to peroxisomes in a Pex19p-dependent manner. However, when peroxisomes are absent or Pex19p is not functional, Pex26p is mistargeted to mitochondria. If a chaperone/import factor is involved in the mitochondrial targeting of hFis1, it would compete with Pex19p for hFis1 binding. If a chaperone/import factor is not involved, it remains to be clarified why hFis1 is not completely routed to peroxisomes (for example, under conditions of massive peroxisome proliferation). It has to be elucidated if targeting is primarily regulated by the level of hFis1 and/or Pex19p in the cytosol. Overexpression of Pex19p had no detectable positive effect on hFis1 targeting to peroxisomes. We strongly assume that other rate-limiting factors of the peroxisomal import machinery are involved. An interesting candidate might be Pex3p, the peroxisomal membrane receptor for Pex19p (44).

TA proteins destined for the peroxisomal membrane were also reported to be initially targeted to the ER and subsequently sorted to peroxisomes by bipartite targeting signals (29–32). We provide evidence that hFis1 is targeted directly to peroxisomes by binding to Pex19p, and that it does not travel via the ER to peroxisomes. ER targeting, which is supposed to occur by default, *i.e.* in the absence of a mitochondrial targeting signal, was only observed after replacing the lysine residues in the C-terminal tail of hFis1.

Very recently, studies on the targeting of TA proteins to peroxisomes have been further complicated by the discovery of a novel vesicular trafficking pathway from mitochondria to peroxisomes, which involves MDVs (34). Thus, another pathway

for hFis1 targeting to peroxisomes might be via mitochondria by a population of MDVs. However, the data obtained by Neuspiel *et al.* (34) are more supportive for a receptor-based peroxisomal targeting of hFis1. First, a subpopulation of MDVs is only targeted to a small population of peroxisomes, whereas hFis1 is targeted in equal amounts to nearly all peroxisomes. Second, hFis1 has not been found to be a prominent cargo of MDVs. We now present evidence that targeting of hFis1 to peroxisomes and mitochondria are independent events.

Information on TA protein targeting to peroxisomes is still limited due to the small number of TA proteins identified and examined (30, 31, 33). Besides hFis1, which has been the first TA protein discovered to localize to both mitochondria and peroxisomes, a second TA protein, Mff, has very recently been discovered (21). Like hFis1, Mff is also supposed to be involved in mitochondrial and peroxisomal division, but might fulfill different functions. Interestingly, there appears to be no obvious Mff homologue in yeast (21). The only known peroxisomal TA proteins in the yeast *Saccharomyces cerevisiae* are Pex15p and Fis1 (20, 33). Using a prediction program for Pex19p binding sites (40, 55), a potential Pex19p binding site (amino acids 170–179) could also be identified in Mff. Whether Mff is indeed capable of binding Pex19p has to be experimentally tested. However, this work and previous findings strongly support a direct, Pex19p-dependent targeting of peroxisomal TA proteins.

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