Dynamin-like Protein 1 Is Involved in Peroxisomal Fission*

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The mammalian dynamin-like protein 1 (DLP1), a member of the dynamin family of large GTPases, possesses mechaanochemical properties known to constrict and tubulate membranes. In this study, we have combined two experimental approaches, induction of peroxisome proliferation by Pex11pβ and expression of dominant-negative mutants, to test whether DLP1 plays a role in peroxisomal growth and division. We were able to localize DLP1 in spots on tubular peroxisomes in HepG2 cells. In addition, immunoblot analysis revealed the presence of DLP1 in highly purified peroxisomal fractions from rat liver and an increase of DLP1 after treatment of rats with the peroxisome proliferator bezafibrate. Expression of a dominant negative DLP1 mutant deficient in GTP hydrolysis (K38A) either alone or in combination with Pex11pβ caused the appearance of tubular peroxisomes but had no influence on their intracellular distribution. In co-expressing cells, the formation of tubulo-reticular networks of peroxisomes was promoted, and peroxisomal division was completely inhibited. These findings were confirmed by silencing of DLP1 using siRNA. We propose a direct role for the dynamin-like protein DLP1 in peroxisomal fission and in the maintenance of peroxisomal morphology in mammalian cells.

Peroxisomes are ubiquitous subcellular organelles that participate in a variety of important catabolic and anabolic functions, including hydrogen peroxide metabolism, the β-oxidation of fatty acids, and the biosynthesis of ether phospholipids (1). Like other subcellular organelles, peroxisomes have the capacity to proliferate and multiply or to be degraded in response to nutritional and environmental stimuli (2). According to the "growth and division model" of Lazarow and Fujiki (3), new peroxisomes form by division and fission of preexisting ones after the import of newly synthesized proteins from the cytosol. Although accumulating evidence suggests that vesicles that originate from the endoplasmic reticulum or other kinds of endomembranes might be involved in the de novo formation of peroxisomes in a multistep process (4–6), the "growth and division model" has been broadly accepted (7). The peroxisomal membrane protein Pex11p appears to be directly involved in the regulation of peroxisomal growth in size and number and, thus, in peroxisomal division. Pex11p-deficient yeast cells contain a small number of "giant" peroxisomes, whereas overexpression results in a high degree of peroxisome proliferation (8, 9). In mammals, three Pex11p molecules, Pex11pα (10, 11), Pex11pβ, and Pex11pγ, have been described, which are supposed to control peroxisome proliferation under induced and basal conditions, respectively (12–14). When overexpressed, Pex11pβ induces a pronounced peroxisome proliferation through a multistep process involving peroxisome elongation and segregation of Pex11pβ from other peroxisomal membrane proteins, followed by peroxisome division (13). Tubulation and fission processes of elongated peroxisomes have also been observed under conditions of rapid cellular growth or stimulation of cultured cells with defined growth factors, fatty acids, or free radicals and have been proposed to contribute to peroxisome proliferation (15–17). At present, however, little information is available on the exact function of such complex tubular or reticular peroxisomal structures, their dynamic behavior, and the molecular machinery required for their formation and division (18).

Proteins of the dynamin family are large GTPases, which have been implicated in tubulation and fission events of cellular membranes, either as a molecular switch or as a pinchase-like mechanoenzyme (19–22). Recent in vitro studies have indicated that conventional dynamin has the ability to tubulate spherical liposomes and, upon GTP hydrolysis, constrict, deform, or sever membrane tubules into discrete vesicles (23–26). The dynamin-like proteins Dnm1 (Saccharomyces cerevisiae), DRP-1 (Caenorhabditis elegans), and mammalian DLP1* are homologues involved in the control of mitochondrial morphology and division (21, 27–30). The mammalian DLP1 is suggested to function in the maintenance of mitochondrial morphology (31–34). It forms a homotetrameric complex similar to dynamin (35) and has been localized to mitochondria but also to other cellular organelles (34, 36, 37). Recently, it has been demonstrated that DLP1 is able to form rings and tubulate membranes in a nucleotide-dependent manner both in living cells and in vitro (38).

In the present study, we have investigated whether DLP1 has an influence on peroxisomal morphology and division. Using Pex11pβ expression to induce tubular peroxisomes as well as peroxisome proliferation, we were able to localize DLP1 in spots on elongated peroxisomes. Expression of a dominant-negative DLP1 mutant deficient in GTP hydrolysis or silencing of DLP1 by siRNA inhibited peroxisomal fission and caused

* This work was supported in part by a grant of the Medizin Stiftung (Marburg, Germany) (to A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: DLP1, dynamin-like protein 1; PMP, peroxisomal membrane protein; WT, wild type; TRITC, tetramethylrhodamine isothiocyanate; GFP, green fluorescent protein; MOPS, 4-morpholinopropanesulfonic acid; siRNA, small interfering RNA.
tubulation of peroxisomes. These findings provide the first evidence suggesting that a dynamin-like protein, DLP1, is involved in peroxisomal fission and in the maintenance of peroxisomal morphology in mammalian cells.

**EXPERIMENTAL PROCEDURES**

dNAs and Antibodies—Wild-type DLP1 (DLP1-WT), DLP1 fused to GFP (DLP1-WT-GFP), and the point mutants GFP-DLP1-K38A and GFP-Dyn2(aa)-K44A were described previously (34, 39). The C-terminally tagged version of Pex11βpmc in pCDNA3 was described in Ref. 13. Rabbit anti-PMP70 (40) and rabbit anti-acyl-CoA oxidase polyclonal antibodies (41) were a gift from Dr. A. Vo (Hamburg, Germany). Rabbit anti-PMP70 (40) and rabbit anti-acyl-CoA oxidase polyclonal antibodies (41) were obtained from Dianova (Hamburg, Germany). The monoclonal anti-tubulin antibodies (41) were a gift from Dr. A. Vo (Hamburg, Germany). Rabbit anti-PMP70 (40) and rabbit anti-acyl-CoA oxidase polyclonal antibodies (41) were obtained from Alexis Corp. (San Diego, CA). The monoclonal anti-tubulin antibody (DM1α) was obtained from Sigma. Species-specific anti-IgG antibodies conjugated to TRITC or fluorescein isothiocyanate were obtained from Dianova (Hamburg, Germany).

**RESULTS**

**DLP1 Localizes to Tubular Peroxisomes**—To investigate the role of DLP1 in peroxisome fission and maintenance of morphology, we took advantage of the human hepatoblastoma cell line HepG2, which has been recently used to propose a general model for peroxisome morphogenesis (16, 48). The peroxisomal compartment in HepG2 cells exhibits marked morphological heterogeneity (15). Elongated and tubular peroxisomes, which are frequently detected in cultured cells, undergo dynamic changes and are observed to divide into spherical organelles (Fig. 1A). Overexpression of the human PEX11β gene alone was sufficient to induce a pronounced proliferation of peroxisomes through a multistep process involving peroxisome elongation (Fig. 1, B and D) and segregation of Pex11β from other peroxisomal membrane proteins (Fig. 1B), followed by peroxisome division (Fig. 1, C and D) (16). Only 6 h after transfection, the peroxisomes in 90% of the Pex11β-pmc-expressing cells displayed an elongated, tubular morphology. Peroxisome tubules declined rapidly in abundance over the following 24–48 h and were replaced by numerous, small spherical peroxisomes (Fig. 1, C and D). In controls (untransfected or vector alone), 30–40% of the cells exhibited tubular peroxisomes after ∼20 h, which is dependent on culture conditions (15, 16). However, elongated peroxisomes in controls were also observed to divide and declined with time in culture, giving rise to spherical organelles (Fig. 1, C and D). These observations indicate that HepG2 cells, and especially those expressing Pex11βpmc provide an excellent model system to study peroxisomal elongation and fission in more detail.

As described previously (36, 34), DLP1 has been localized to mitochondria, endoplasmic reticulum tubules and other, yet unidentified cellular organelles in mammalian cells. As shown in Fig. 2, affinity-purified antibodies to DLP1 stain punctate vesicular structures in HepG2 cells that are concentrated at the perinuclear region and appear to form linear arrays (Fig. 2, A and B). The intensity of the fine punctate staining pattern was increased by transfection of HepG2 cells with either a wild type DLP1 construct (Fig. 2B) or expression of a wild type DLP1-GFP fusion protein (Fig. 2C). The expression of the constructs did not influence the normal intracellular distribution of DLP1. Similar observations have been made in other mammalian cells (36, 34). To determine whether DLP1 associates with multiplying peroxisomes, HepG2 cells expressing Pex11βpmc were fixed, permeabilized, and double-stained with antibodies to DLP1 and to the Myc tag of Pex11βpmc. In addition, HepG2 cells were co-transfected with Pex11βpmc and assayed were run with a recording spectrophotometer (Uvikon S10) (Kontron, Munich, Germany) or Beckman model 24).

**Gel Electrophoresis and Immunoblotting**—Protein samples were separated by SDS-PAGE, transferred to nitrocellulose (Schleicher and Schuell) using a semidyel apparatus and analyzed by immunoblotting. Immunoblots were processed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham Biosciences). For quantification, immunoblots were scanned and processed using Picas software.

**Quantitation and Statistical Analysis of Data**—For quantitative evaluation of peroxisome morphology, 100–200 cells per coverslip were examined and categorized as cells with tubular (2–5 μm in length; Figs. 1B and 4D), rod-shaped (0.5–1 μm; Figs. 1A and 4B), or spherical peroxisomes (0.1–0.3 μm; Fig. 1A) as described (15–17). Cells co-expressing Pex11βpmc and GFP-DLP1-K38A were usually filled with extremely elongated (up to 15 μm) peroxisomes (hypertubulation). Usually, 3–5 coverslips per preparation were analyzed, and 4–8 independent experiments were performed. Significant differences between experimental groups were detected by analysis of variance for unpaired variables using Microsoft Excel. Data are presented as means ± S.D., with an unpaired t test used to determine statistical differences. p values <0.05 are considered as significant, and p values <0.01 are considered as highly significant.

**Isolation of Peroxisomes**—Peroxisomes were isolated from rat liver according to a protocol described previously (43). For double-labeling experiments, cells transfected with GFP constructs were incubated with rabbit anti-PMP70 (40) or other peroxisomal marker proteins and subsequently with goat anti-rabbit IgG conjugated to TRITC. For visualization of Pex11βpmc, cells were labeled with anti-Myc (TRITC) antibodies. Samples were examined using a Leitz Diaplan (Leica, Wetzlar, Germany) or an Axiovert 100 microscope (Carl Zeiss, Jena, Germany) equipped with the appropriate filter combinations and photographed on a Leica TCS MP confocal microscope equipped with the appropriate filter combinations and photographed on Eastman Kodak Co. TMY film or digitalized. Confocal images (150-nm sections) were captured with a Leica TCS MP confocal microscope (Leica Microsystems, Bensheim, Germany) with appropriate spectrometer settings for each fluorophor. Digital images were optimized for contrast and brightness using Micrograph Picture Publisher software.

**Isolation of Peroxisomes**—Peroxisomes were isolated from rat liver according to a protocol described previously (43). For some experiments, peroxisomes were isolated from the livers of adult male Wistar rats (Charles River, Sulzfeld, Germany), which were fed with the potent peroxisome proliferator bezafibrate (41) (Roche Molecular Biochemicals). Briefly, one liver was homogenized (1 stroke, 2 min, 1000 rpm) using a Potter S homogenizer (Braun, Melsungen, Germany) in ice-cold 5 mM MOPS, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.1% ethanol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM e-aminoacaproic acid, and 0.2 mM dithiothreitol. After subcellular fractionation, a crude peroxisomal fraction (light mitochondrial fraction D) was sedimented into exponentially shaped OptiPrep (Axis-Shield, Oslo, Norway) gradients (40). The highly purified peroxisomal fraction was collected and purified by centrifugation at 18000 × g for 30 min at 56°C (47). Protein was determined using standard procedures. All
Fig. 1. Pex11pβ expression induces proliferation of peroxisomes in HepG2 cells. A, HepG2 cells were processed for indirect immunofluorescence using an antibody specific for PMP70, a peroxisomal membrane protein. Note the elongated peroxisomes in the cell on the left, in contrast to their segmented appearance in the cell on the right. The arrows point to peroxisomes that are supposed to divide. B–D, overexpression of Pex11pβmyc induces peroxisome elongation prior to fission and proliferation. B, HepG2 cells were transfected with Pex11pβmyc, a Myc-tagged version of a peroxisomal membrane protein, and labeled with antibodies specific for PMP70 (red) and the Myc epitope tag (green). Peroxisome proliferation coincides with the elongation of peroxisomes and the segregation of Pex11pβmyc from PMP70 (or peroxisomal matrix proteins). The arrow points to an elongated peroxisome with multiple, alternating bands of Pex11pβmyc compared with controls (cell on the left). D, peroxisome morphology at different time points after transfection. time (h), time after transfection; squares, control cells (vector alone or untransfected); triangles, Pex11pβmyc-expressing cells; black, tubular peroxisomes; white, rodlike and spherical peroxisomes; N, nucleus; bars, 10 μm (A and C) or 2.5 μm (B).

and DLP1-WT-GFP. Interestingly, DLP1-positive structures were found to distribute along the length of elongated peroxisomes (Fig. 2, D–H). DLP1 was found to align along some tubular peroxisomes in spots and was associated with the tips of some tubules (Fig. 2, D and E). In addition, other nonperoxisomal intracellular structures, presumably mitochondria, were positive for DLP1. Co-localization with tubulo-reticular peroxisomes was confirmed by confocal laser-scanning microscopy. DLP1 (Fig. 2F) or DLP1-WT-GFP (Fig. 2H) were found to form small patches along the tubular structures and at the tips. Similar observations were made in untransfected controls; however, co-localization was less frequent (Fig. 2G). In contrast, colocalization with peroxisomes was not observed when an antibody to dynamin II, an ubiquitously expressed dynamin isoform, was used (not shown).

Fig. 2. DLP1 localizes to elongated peroxisomes in Pex11pβ-expressing HepG2 cells. A–C, DLP1 is associated with numerous punctate vesicular structures in HepG2 cells. Immunofluorescence microscopy of control cells (A) or HepG2 cells transfected with DLP1-WT (B), stained with affinity-purified antibodies to DLP1. C, HepG2 cells transfected with DLP1-WT-GFP. D–H, HepG2 cells expressing Pex11pβmyc only (D–F) or Pex11pβmyc in combination with DLP1-WT-GFP (H) and untransfected controls (Con) (G) were processed for indirect immunofluorescence using antibodies to the Myc epitope tag (D, F, H), to PMP70 (G), and to DLP1 (E–G). F, G, and H, confocal images showing localization of DLP1 (red) (F and G) or DLP1-WT-GFP (green) (H) along elongated peroxisomes in Pex11pβmyc-expressing cells (F and H) and untransfected controls (G). The arrows in A point to linear arrays of DLP1 staining; arrows in D–H point to regions of co-localization. Bars, 10 μm (A–C) or 2.5 μm (D–H).
acyl-CoA oxidase and catalase, mitochondrial cytochrome c and peroxidase. The positions of molecular mass markers (in kDa) are indicated on the gel. Additionally, two sets of parallel gels, blotted onto nitrocellulose membranes, and incubated with anti-AOX (Beza Br, rat brain homogenate (5 µg/lane); Cyt, cytosolic fraction (10 µg/lane); Po, peroxisomes; Mit, mitochondria; Mic, microsomes; Lys, lysosomes. C, highly purified peroxisomes from controls (−) and rats treated with the peroxisome proliferator bezafibrate (+) (Beza) were isolated, separated on 12.5% acrylamide gels, blotted onto nitrocellulose, and incubated with antibodies to peroxisomal acyl-CoA oxidase (AOX), catalase (CAT), or DLP1. Equal amounts of protein (acyl-CoA oxidase and catalase, 4 µg/lane; DLP1, 40 µg/lane) were loaded onto the gels. For DLP1, immunoblots loaded with different amounts of protein (30–50 µg/lane) were quantitated and are expressed as means ± S.D. Proteins were detected by ECL using streptavidin-peroxidase. The positions of molecular mass markers (in kDa) are indicated on the right. A–C, three subunits of acyl-CoA oxidase with molecular masses of 72, 52, and 20.5 kDa. Similar results were obtained in three independent experiments.

To investigate whether the amount of DLP1 in the peroxisomal fraction was increased after the induction of peroxisome proliferation by pharmacological compounds, rats were treated with the potent peroxisome proliferator bezafibrate (41). Rodents are known to respond to a variety of xenobiotics with a massive peroxisome proliferation, whereas primates are “low responders.” Peroxisome proliferation is usually accompanied by an induction of peroxisomal β-oxidation enzymes and an increase in peroxisome size and number. After treatment of rats with bezafibrate, an induction of acyl-CoA oxidase, a key enzyme of peroxisomal β-oxidation, was observed in a highly purified peroxisomal fraction when compared with untreated controls (Fig. 3C). In contrast, catalase is not induced or is only slightly induced (Fig. 3C). The determination of marker enzyme activities in the peroxisomal fractions isolated from bezafibrate-treated rats revealed a purity similar to the controls (not shown). In electron microscopic studies, an increase in the size and number of peroxisomes was also observed (41). After immunoblotting, DLP1 was found to be increased about 2-fold in the peroxisomal fractions obtained from bezafibrate-treated animals when compared with controls (Fig. 3C). Similar results were obtained when rat Fao cells were treated with the potent peroxisome proliferator ETYA (not shown).

**DLP1 Mutants Cause Morphological Changes of Peroxisomes—**To examine whether a mutated DLP1 affects peroxisome morphology, we transfected HepG2 cells with a DLP1-GFP construct harboring a lysine-to-alanine (K38A) mutation in GTP binding element 1 (34). It has been shown recently that recombinant DLP1-K38A protein is capable of binding GTP but is deficient in its hydrolysis due to reduced GTPase activity (38). We transiently co-transfected HepG2 cells with Pex11p and the mutated GFP-DLP1-K38A. Cells were immunostained 24 h after transfection with antibodies to Pex11p (Fig. 4). In contrast to the normal distribution of DLP1, GFP-DLP1-K38A assembled into large cytoplasmic aggregates in addition to associating with punctate vesicular structures (Fig. 4; see also Ref. 34). Recent ultrastructural studies revealed that the large cytoplasmic aggregates are composed of tubular membrane clusters that are coated with DLP1 in a periodic manner (38). In cells co-expressing Pex11p and GFP-DLP1-K38A, a pronounced elongation of peroxisomes (“hypertubulation”) was observed (Figs. 4, A–C, and 6). The cytoplasm of these cells was filled with numerous, largely elongated peroxisomes. They usually ranged in length from 2 to 8 µm, but tubular forms measuring 10–15 µm were also found. Interestingly, extended tubulo-recticular networks of peroxisomes were frequently observed when confocal laser-scanning microscopy was applied, suggesting that fusion of elongated peroxisomes was promoted (Fig. 5A). Furthermore, some of the DLP1-positive cytoplasmic aggregates were found to localize to other cell organelles and highly pure (43). The resulting fractions were analyzed by immunoblotting with an anti-DLP1 antibody, which has been characterized previously on rat liver subcellular fractions (36). The DLP1-specific antibody detected a single band of the expected size (80 kDa) in rat liver cytosol and in a rat brain homogenate (Fig. 3B). Brain DLP1 ran slightly higher on SDS-PAGE than DLP1 from the cytosolic fraction or from other rat tissues (36). Whereas DLP1 was prominent in the cytosolic fraction, there was a small but reproducible amount of DLP1 in the highly purified peroxisomal fraction. In addition, a small amount was detected in the mitochondrial and the microsomal fractions under our experimental conditions (Fig. 3B). In contrast, DLP1 was absent from a lysosomal fraction, which has been suggested previously (36).

**Intact centrifugation, was further purified on exponentially shaped OptiPrep gradients (Fig. 3A). Peroxisomes were mainly recovered in fractions 2–4 with the mean equilibrium density of 1.24 g/ml. Mitochondria mostly band in a density range of about 1.15 g/ml (fractions 12 and 13), and microsomes (density of about 1.11 g/ml) were predominantly found in fractions 14 and 15. Some activity of the peroxisomal marker enzyme catalase was also found in the uppermost gradient fractions, reflecting the fragmentation of the fragile peroxisomes during centrifugation (Fig. 3A). The determination of marker enzyme activities in the main organelle fractions revealed no contamination of the peroxisomal fraction with mitochondria, microsomes, or lysosomes (Table I). These data demonstrate that the isolated peroxisomal fraction is well separated from other cell organelles and highly pure (43). The resulting fractions were analyzed by immunoblotting with an anti-DLP1 antibody, which has been characterized previously on rat liver subcellular fractions (36). The DLP1-specific antibody detected a single band of the expected size (80 kDa) in rat liver cytosol and in a rat brain homogenate (Fig. 3B). Brain DLP1 ran slightly higher on SDS-PAGE than DLP1 from the cytosolic fraction or from other rat tissues (36). Whereas DLP1 was prominent in the cytosolic fraction, there was a small but reproducible amount of DLP1 in the highly purified peroxisomal fraction. In addition, a small amount was detected in the mitochondrial and the microsomal fractions under our experimental conditions (Fig. 3B).

In contrast, DLP1 was absent from a lysosomal fraction, which has been suggested previously (36).
properties of the purified organelle fractions

The relative specific activity (RSA) was determined with respect to the crude peroxisomal fraction. Values given are means ± S. D. The data are from three independent experiments. CytoCox, cytochrome c oxidase; β-Gluc, β-glucuronidase.

| Fraction (No.) | Catalase | CytoCox | Esterase | β-Gluc |
|----------------|----------|----------|----------|--------|
| Peroxisomes (3) | 4.83 ± 1.1 | 0.02 ± 0.1 | 0 | 0.47 ± 0.2 |
| Mitochondria (12) | 1.7 ± 0.5 | 1.2 ± 0.5 | 0.69 ± 0.3 | 0.82 ± 0.14 |
| Microsomes (15) | 0.16 ± 0.2 | 0.36 ± 0.2 | 1.13 ± 0.45 | 0.86 ± 0.2 |
| Lysosomes (17) | 0.5 ± 0.2 | 0.33 ± 0.2 | 1.86 ± 0.7 |

Fig. 4. Inhibition of DLP1 function induces “hypertubulation” of peroxisomes in Pex11β-expressing cells. HepG2 cells were co-transfected with Pex11β-myc/GFP-DLP1-K38A (A–D), with Pex11β-myc/GFP-Dyn2-K44A (G and H), or with GFP-DLP1-K38A only (E and F) and immunostained with antibodies to the Myc tag of Pex11β (A, C, D, and G) or the peroxisomal membrane protein PMP70 (E). The corresponding GFP fluorescence of DLP1-K38A (B and F) and Dyn2-K44A (H) is shown on the right. Higher magnification images of boxed regions in A are shown in C and D. Note the pronounced elongation of peroxisomes in DLP1-K38A co-expressing cells. Asterisks, co-expressing (A, B, G, and H) or transfected cells (E and F). Bars, 10 μm.

Fig. 5. Co-expression of Pex11β-myc and GFP-DLP1-K38A promotes the formation of reticular peroxisomal networks. A and B, confocal images of HepG2 cells co-expressing Pex11β-myc (red) and GFP-DLP1-K38A (green). Cells were immunostained with an anti-Myc antibody. A, higher magnification view of a tubulo-reticular peroxisomal network induced by co-expression. B, note the association of GFP-DLP1-K38A-positive cytoplasmic aggregates with the tips of elongated peroxisomes. The arrows in B point to regions of co-localization. Bars, 2.5 μm.

The striking induction of elongated tubulo-reticular peroxisomes in cells expressing GFP-DLP1-K38A prompted us to investigate the influence on peroxisomal fission more carefully in a time course experiment. Besides HepG2 cells, COS-7 cells were used, which possess an elaborate peroxisomal compartment (18). Cells co-expressing Pex11β-myc and GFP-DLP1-K38A were immunostained 24 and 48 h after transfection with antibodies to Pex11β-myc, and the cells were quantitated according to their different peroxisomal forms (Fig. 6). In cells expressing Pex11β-myc only or Pex11β-myc in combination with either DLP1-WT or DLP1-WT-GFP, a typical decrease in tubular peroxisomes was noted after 48 h, whereas a prominent increase in cells containing spherical peroxisomes was observed (Figs. 1, C and D, and 6). Furthermore, segmented tubules, presumably in division, were detected (see Fig. 1B).
However, in cells expressing both Pex11p/myc and GFP-DLP1-K38A, tubular peroxisomes were found to accumulate. After 48 h, nearly all co-expressors contained largely elongated peroxisome tubules, whereas cells with spherical peroxisomes were missing completely (Fig. 6). Segmented tubules were not observed any more, but reticular networks of peroxisomes were detectable (Fig. 5A). In cells co-expressing GFP-Dyn2(aa)-K44A and Pex11p/myc, tubular peroxisomes were slightly increased after 48 h compared with Pex11p/myc alone (Fig. 6, A and B). The accumulation of elongated peroxisomes was more pronounced in COS-7 than in HepG2 cells. However, tubular peroxisomes in these cells were usually less elongated and less frequent than in DLP1-K38A/Pex11p/myc expressors. Although less obvious in COS-7 cells, the HepG2 cells still kept their ability to generate spherical peroxisomes by fission of elongated ones (Fig. 6A). We therefore assume that the enrichment of elongated peroxisomes in Dyn2-K44A/Pex11p/myc expressors may be due to an additional proliferative stimulus exerted by the inhibition of Dyn2 function and not to a direct role in peroxisome division.

Next, we addressed whether the expression of GFP-DLP1-K38A alone was able to influence tubulation and fission of peroxisomes in HepG2 and COS-7 cells (Fig. 7). Cells were immunostained 24 and 48 h after transfection with antibodies to PMP70, a peroxisomal membrane protein, and quantitated as described in Fig. 6. Note the accumulation of elongated peroxisomes (rods and tubules) in cells expressing GFP-DLP1-K38A only were immunostained 24 and 48 h after transfection with antibodies to PMP70, a peroxisomal membrane protein, and quantitated as described in Fig. 6. The data are from six independent experiments and are expressed as means ± S.D. (*, p < 0.01; #, p < 0.05). For qualitative evaluation, cells were categorized as cells with spherical (s), rod-shaped (r), or tubular (t) peroxisomes.
co-expression with Pex11pβmyc, a pronounced “hypertubulation” was not observed (see Fig. 4, E and F). However, a significant shift toward more elongated peroxisomal forms (rods and tubules) was detected 24 h after transfection, which was more pronounced after 48 h, when compared with control cells (untransfected, DLP1-WT, or DLP1-WT-GFP) (Fig. 8). Furthermore, an accumulation of elongated peroxisomal forms was observed, whereas the majority of the control cells contained spherical peroxisomes after 48 h (Fig. 7). Dyn2 (K44A)-expressing cells were indistinguishable from control cells when peroxisomal forms were compared (not shown). These findings indicate that mutated DLP1 (K38A) has the capacity to cause the appearance of tubular peroxisomal membranes even in the absence of a proliferative stimulus, although elongation appears to be more pronounced in the presence of a stimulus. We conclude from these data that DLP1 is involved in the fission process of peroxisomes.

**Inhibition of DLP1 Function Does Not Change Peroxisomal Distribution**—It has been reported that the inhibition of DLP1 function results in a change of the normal distribution of mitochondria (31, 32, 34, 52). Similar observations were made in DLP1 mutant-expressing HepG2 and COS-7 cells, where mitochondria were often found to be clustered and wrapped around the nucleus when compared with controls (Fig. 8, A and B). In contrast, the uniform intracellular distribution of peroxisomes was not affected by the expression of GFP-DLP1-K38A, either alone (Fig. 8C) or in combination with Pex11pβmyc (Fig. 4). The tubular peroxisomes observed in cells expressing GFP-DLP1-K38A only or in combination with Pex11pβmyc are not stained by antibodies to mitochondrial or endoplasmic reticular marker proteins but are positive for other peroxisomal marker proteins (e.g. PMP70) (13). Although similar in morphology to elongated mitochondria, tubular peroxisomes are thinner and less elongated and do not exhibit bulbous structures (Fig. 8, compare A and C). Furthermore, their intracellular distribution remains unchanged after GFP-DLP1-K38A expression, which is in contrast to tubular mitochondria.

**Silencing of DLP1 by siRNA Causes Tubulation and Aggregate Formation of Peroxisomes**—To verify the results obtained with the mutated, nonfunctional DLP1 (K38A), we conducted RNA interference experiments to “knock down” the expression of DLP1. Silencing of DLP1 in HepG2 cells was mediated by transfecting the cells with 21-nucleotide siRNA duplexes that targeted human DLP1. Efficient siRNA-mediated gene silencing in mammalian cells has recently been reported by Tuschl and co-workers (53). HepG2 cells were processed for immunofluorescence 48 h after transfection using antibodies directed to DLP1 and catalase. As shown in Fig. 9, the expression of DLP1 was specifically reduced by the cognate siRNA duplex. However, a fine punctate and diffuse cytoplasmic staining was still visible (Fig. 9D). A reduction of DLP1 after transfection was also observed in immunoblots of cell homogenates (Fig. 9F). In controls treated with buffer or a noneffective siRNA, the expression of DLP1 was not reduced (Fig. 9, B and F). We also obtained an siRNA for DLP1, which was not effective in silencing DLP1. The peroxisomes in these controls were overwhelmingly spherical, and tubular peroxisomes were usually completely absent after 3 days in culture (Fig. 9A; see also Fig. 1). Interestingly, the peroxisomes in transfected cells became highly elongated (40–60% of the cell population) and formed tubulo-reticular aggregates. The elongated peroxisomes induced by siRNA often had a segmented appearance but were not observed to separate into spherical organelles. Elongation of mitochondria and clustering around the nucleus was also visible after siRNA-mediated DLP1 silencing (not shown). These observations further confirm a role for DLP1 in peroxisomal fission and in the maintenance of peroxisomal morphology in mammalian cells.

**DISCUSSION**

In this study, we have combined two experimental approaches, induction of peroxisome proliferation by Pex11pβ...
and expression of dominant negative mutants, to examine whether DLP1 plays an important role in peroxisomal fission and in the maintenance of peroxisomal morphology in mammalian cells.

A Direct Role for Pex11pβ in Peroxisomal Fission?—The growing recognition of the dynamic nature of the peroxisomal compartment in eukaryotic cells has inspired the query into the investigation of the cellular machinery that mediates such a complex behavior. At present, however, little information is available on the molecular components involved in peroxisome growth and division. The characterization of Pex11p in both yeast and mammalian cells and in trypanosomes has led to the proposal that it is directly involved in the regulation of peroxisome growth in size and number (8, 9, 11, 13, 54). Since cells that lack Pex11p accumulate a few large peroxisomes in contrast to Pex11p overexpressors, which contain numerous small peroxisomes, it has been assumed that Pex11p is involved in the fission process. In support of a more direct role of Pex11p in peroxisomal division, it has been shown that rat Pex11pα binds coatomer in vitro by its cytoplasmically exposed C-terminal dilysine motif. Consequently, recruitment of coatomer by Pex11pα has been proposed to initiate vesiculation or budding of peroxisomes (11). However, the dilysine motif is not conserved in other Pex11p homologues, and coatomer does not bind to yeast Pex11p or human Pex11pβ. Furthermore, mutations of the C terminus of Pex11p were not found to affect its function in peroxisome division (55), and studies on PEX3-mediated peroxisome biogenesis suggest that it can proceed independently of both COPI and COPII (56). Here we have demonstrated that peroxisomal fission requires a functional DLP1, indicating that Pex11pβ, which is supposed to control constitutive peroxisome abundance in mammals (13), is not directly mediating the vesiculation process of the organelle. In support of a secondary, indirect role of Pex11p in peroxisomal division, van Roermund et al. (57) proposed a function for yeast ScPex11p1 in medium-chain fatty acid β-oxidation rather than in peroxisomal fission. They favor the idea that ScPex11p1 is involved in the transport of fatty acids or cofactors across the peroxisome membrane and suggest that it is part of a signaling event that modulates peroxisome proliferation. In contrast, Li and Gould (58) showed that Pex11p α promotes peroxisome division in the absence of peroxisomal metabolic activity. They suggest that Pex11 proteins play a direct role in peroxisome division and that their loss inhibits peroxisome metabolism indirectly. If Pex11pβ is more directly involved in the fission process, it must act upstream of DLP1. But what could be its function in peroxisome division? The rapid tubulation of the peroxisomal membrane induced by Pex11pβ overexpression (alone or in combination with a DLP1 mutant) might be indicative of a change in membrane lipid composition or a modification of peroxisomal lipids mediated by Pex11pβ function. Since the association of DLP1 to peroxisomal membranes was more pronounced after Pex11pβ expression, we speculate that a membrane-modifying activity of Pex11pβ could initiate and/or favor the binding of DLP1 and other factors of the fission machinery to the peroxisomal membrane.

A Role for DLP1 in Peroxisomal Division—The function of mammalian DLP1 is still a matter of debate. DLP1 has been shown to participate in mitochondrial morphogenesis and fission in mammalian cells and yeast (27, 28, 31, 32, 34). However, DLP1 is also found to occur at cytoplasmic sites other than mitochondria, including microtubules and endoplasmic reticulum cisternae (36, 34, 38), and evidence for the action in other cellular processes has been reported (34, 37). The data presented in this study support an additional role for DLP1 in peroxisomal division. A direct role of DLP1 in peroxisomal fission is substantiated by the localization of DLP1 in spots on peroxisomal tubules. The DLP1 spots were detected by immunofluorescence of endogenous DLP1 with a DLP1-specific antibody but also with GFP-tagged DLP1. Since the segmentation of elongated peroxisomes has been connected with peroxisomal fission (13), these morphological observations are likely to be consistent with a direct role of DLP1 in peroxisomal division. Furthermore, DLP1 was found to be associated with highly purified peroxisomes from rat liver. Based on our biochemical results, it is unlikely that this association is due to contamination of the peroxisomal fraction with mitochondria or microsomes. Interestingly, the association of DLP1 to peroxisomes was increased under proliferative conditions induced by the peroxisome proliferator bezafibrate. In addition, the localization of DLP1 to tubular peroxisomes was more pronounced after expression of Pex11pβ. Expression of Pex11pβ has been shown to result in rapid elongation of peroxisomes, followed by segregation of peroxisomal proteins and formation of numerous small peroxisomes (13), thus representing a strong stimulus for peroxisome proliferation. These observations make sense, because a recruitment of DLP1 to peroxisomes would be required during rapid growth and division of the peroxisomal compartment. It might also explain why an association of DLP1 with peroxisomes has not yet been noted in other studies.

A most striking observation was the complete inhibition of peroxisome division after expression of the mutated DLP1-K38A in conjunction with Pex11pβ. The characteristic appearance of small peroxisomes was completely inhibited by co-expression of DLP1-K38A, and further tubulation of the organelles appeared as large tubular structures. The expression of DLP1 was also observed after expression of DLP1-K38A alone independently of Pex11pβ expression, although the organelles appeared to be less elongated. The expression of a mutated dynamin II had a weak effect on peroxisomal morphology but did not inhibit peroxisomal division in HepG2 cells. These observations were confirmed by siRNA-mediated silencing of DLP1 and are consistent with a direct role of DLP1 in peroxisomal fission.

In support of this, Hoepfner et al. (59) have recently demonstrated that in yeast lacking the dynamin-related protein Vps1p, the number of peroxisomes was reduced, and peroxisomes appeared as large tubular structures. The authors suggest that Vps1p may be involved in peroxisome fission and consequently in the regulation of peroxisome abundance in yeast. Interestingly, peroxisomes in vps1Δ cells were still able to divide, but peroxisome morphology and number was unaffected in cells lacking Dnm1p. Both yeast Vps1p and Dnm1p share about 42% homology with mammalian DLP1 (36). Based on a common function in mitochondrial division (21, 27, 28), it has been inferred that Dnm1p is the yeast homologue to DLP1, but this is still a matter of debate. Whether DLP1 can be considered a true mammalian homologue of Vps1p and Dnm1p and whether the mechanism of peroxisome division is completely comparable between yeast and mammalian cells has to be elucidated.

How Does DLP1 Tubulate and Divide Peroxisomal Membranes?—It has been demonstrated recently that DLP1-K38A mutant protein was able to bind but not hydrolyze GTP, which resulted in an increased affinity for membranes (38). Furthermore, recombinant DLP1 was capable of forming oligomeric protein ring structures in the presence of GTPyS that were found to deform liposomes into tubules. These findings demonstrated that, despite the limited homology to conventional dynamins (35%), DLP1 tubulates and constricts cytoplasmic...
membranes in a similar manner. We therefore conclude that hydrolysis of GTP by DLP1 is required for proper fission of peroxisomes. For conventional dynamin, which is involved in receptor-mediated endocytosis, it has been shown that it is a membrane-active molecule capable of penetrating into the acyl chain region of membrane lipids and that local lipid metabolism can influence dynamin-lipid interactions (60). In contrast to dynamin, DLP1 lacks a pleckstrin homology or proline-rich domain, known to interact with acidic phospholipids, but was capable of deforming phosphatidylycerine-containing liposomes into tubules (38). Whether the specific interaction of DLP1 with the peroxisomal membrane is mediated by lipids and/or requires other cytosolic or peroxisomal membrane proteins has to be elucidated.

Although the exact mechanism of DLP1 action is unclear at present, it might act as a "peroxisomal pinchase" either by constricting or destabilizing the peroxisomal membrane, thus leading to peroxisomal fission. Whether members of the dynamin family act as mechanochemical enzymes (pinchase model) or function as a molecular switch regulating downstream effectors of membrane fission (molecular switch model) is still a matter of debate (61). The striking similarities between mitochondrial and peroxisomal division and morphogenesis in mammalian cells may point to a similar, perhaps general, mechanism of intracellular membrane fission mediated by DLP1. We therefore favor a model of peroxisome fission similar to the one proposed for mitochondrial division (29, 21, 30). It is likely that dynamin-related GTPases act together with accessory proteins (e.g. cytoskeletal motor proteins, lipid-modifying enzymes) and the local lipid composition in the membrane to mediate constriction and final scission of peroxisomes.

Acknowledgments—We thank Drs. S. J. Gould (The Johns Hopkins University) and A. Volkl (University of Heidelberg) for providing antibodies and cDNA constructs, G. Schneider and W. Sperling (University of Marburg) for excellent technical assistance, and V. Kramer (University of Marburg) for help with the photographic work and image processing.

REFERENCES

1. van den Bosch, H., Schutgens, R. B., Wanders, R. J., and Tager, J. M. (1992) Annu. Rev. Nutr. 11, 157–175.
2. Reddy, J. K., and Mannhaerts, G. P. (1994) Annu. Rev. Nutr. 14, 343–370.
3. Lazarov, P. B., and Fujiki, Y. (1985) Annu. Rev. Cell Biol. 1, 489–530.
4. Waterham, H. R., Titorenko, V. I., Swaving, G. J., Harder, W., and Veenhuis, M. (1993) EMBO J. 12, 4785–4794.
5. South, S. T., and Gould, S. J. (1999) J. Cell Biol. 144, 255–266.
6. Fujiki, Y., and Kinkin, Y. (1974) Biochem. Biophys. Res. Commun. 52, 529–533.
7. Jaffe, E. S., and Khanna, S. (1991) Trends Biochem. Sci. 16, 257–263.
8. Nakata, T., Takemura, R., and Hirokawa, N. (1993) J. Cell Sci. 105, 1–5.
9. Cook, T. A., Urrutia, R., and McNiven, M. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 91, 644–648.
10. Urrutia, R., and McNiven, M. A. (1993) J. Cell Biol. 128, 1279–1286.
11. Hoepfner, D., van den Berg, M., Philippsen, P., Tabak, H. F., and Hettema, J. M. (1999) Eur. J. Cell Biol. 78, 125–129.
