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Peroxisomal membrane proteins are properly targeted to peroxisomes in the absence of COPI- and COPII-mediated vesicular transport

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SUMMARY

The classic model for peroxisome biogenesis states that new peroxisomes arise by the fission of pre-existing ones and that peroxisomal matrix and membrane proteins are recruited directly from the cytosol. Recent studies challenge this model and suggest that some peroxisomal membrane proteins might traffic via the endoplasmic reticulum to peroxisomes. We have studied the trafficking in human fibroblasts of three peroxisomal membrane proteins, Pex2p, Pex3p and Pex16p, all of which have been suggested to transit the endoplasmic reticulum before arriving in peroxisomes. Here, we show that targeting of these peroxisomal membrane proteins is not affected by inhibitors of COPI and COPII that block vesicle transport in the early secretory pathway. Moreover, we have obtained no evidence for the presence of these peroxisomal membrane proteins in compartments other than peroxisomes and demonstrate that COPI and COPII inhibitors do not affect peroxisome morphology or integrity. Together, these data fail to provide any evidence for a role of the endoplasmic reticulum in peroxisome biogenesis.

Key words: Membrane biogenesis, Protein targeting, Vesicular transport, Peroxisome biogenesis, Coatomer

INTRODUCTION

Eukaryotic cells contain numerous subcellular organelles whose specialized functions require distinct protein compositions. These proteins are either synthesized on membrane-bound polysomes at the endoplasmic reticulum (ER) and sorted to their final destinations by vesicular transport or they are synthesized on free polysomes and imported directly from the cytosol into organelles such as mitochondria, chloroplasts and peroxisomes (Rothman and Wieland, 1996; Schatz and Dobberstein, 1996). Peroxisomal matrix proteins are recruited after synthesis in the cytosol by two soluble receptors, Pex5p and Pex7p, which specifically interact with the peroxisomal targeting signals (PTSs) on the newly synthesized proteins. Both act as mobile receptors that select their PTS ligands in the cytosol and deliver them to a membrane-bound translocation machinery for further transport into the peroxisomal matrix (Erdmann et al., 1997; Hettema et al., 1999; Subramani, 1998). Trafficking of peroxisomal membrane proteins (PMPs) is much less well understood and currently a matter of controversy. One model proposes that all PMPs are synthesized on free polysomes and directly inserted into the peroxisomal membrane (Lazarow and Fujiki, 1985). For at least two PMPs, PMP22 and PMP70, there is experimental evidence for a direct insertion pathway (Diestelkötter and Just, 1993; Imanaka et al., 1996). If true, this would imply that peroxisomes are autonomous organelles, like mitochondria, that multiply by growth and division of preexisting peroxisomes. However, several recent observations have challenged this model and involve the ER as a transitory compartment in peroxisome biogenesis. First, overexpression of wild-type and modified versions of the PMPs Pex3p and Pex15p (either truncated or fusion proteins) resulted in aberrant ER morphology and accumulation of the overproduced proteins in or at the ER (Baerends et al., 1996; Elgersma et al., 1997; Kammerer et al., 1998). Second, peroxisome biogenesis could be partially inhibited by brefeldin A (BFA), a fungal toxin that interferes with COPI-coated-vesicle formation, or by mutation of the ε subunit of COPI (Passreiter et al., 1998; Salomons et al., 1997). Third, the PMPs Pex2p and Pex16p were reported to be *N*-glycosylated in the yeast *Yarrowia lipolytica* (Titorenko and Rachubinski, 1998a). These observations suggest that a subset of PMPs might be targeted first to the ER and from there to peroxisomes by vesicle-mediated transport (Kunau and Erdmann, 1998; Titorenko et al., 2000; Titorenko and Rachubinski, 1998b). If this model were true, it would drastically change our view of peroxisome biogenesis. Instead of being autonomous, self-multiplying organelles, they would be part of the vacuolar compartment of the cells, and the ER would be the source of nascent peroxisomes.

To understand how peroxisomes are formed, either from pre-existing peroxisomes or from the ER, it is thus essential to know the intracellular pathway taken by PMPs. Here, we show that peroxisomal targeting of three PMPs, Pex2p, Pex3p and Pex16p, is not affected by inhibitors of COPI- and COPII-mediated vesicle transport. Moreover, we were unable to detect these PMPs in the ER or any other non-peroxisomal compartment at early times after synthesis. We also show that peroxisome morphology and integrity are not affected by

prolonged incubation with inhibitors of COPI and COPII. Our studies fail to provide any evidence for a role for COPI or COPII in peroxisome biogenesis or PMP targeting and contradict some of the experimental evidence put forward for a role of the ER in peroxisome biogenesis.

MATERIALS AND METHODS

Cell culture, microinjection and immunofluorescence

Cultured primary skin fibroblasts used in this study were from a control subject. Cells were grown in Ham F10 (Gibco BRL, Gaithersburg, MD) supplemented with 10% foetal calf serum (Gibco BRL) and penicillin/streptomycin (100 μg ml^{-1}) under 5% CO_2. Between 24 and 36 hours before microinjection, the cells were plated onto microinjection grids (CELLocate coverslips-square size 175 μm, Eppendorf). The needles were made using PB-7 micropipette puller (Narihage Co., Tokyo, Japan). DNA was microinjected in sterile water at a concentration of approx. 0.05 mg ml^{-1}. In coinjection experiments, plasmids expressing mutant Sar1p and green-fluorescent-protein-tagged (GFP-tagged) PMPs were mixed in a ratio of 3:1 and injected at a concentration of 0.2 mg ml^{-1}. Approximately 200 cells were injected per experiment, ~20% of which survived injection and gave rise to detectable expression. Brefeldin A (BFA; Sigma) was used at a concentration of 2 μg ml^{-1} and was added 20 minutes before injection. After injection, cells were maintained in BFA. At different time points after injection, cells were fixed and processed for indirect immunofluorecence as described previously (Motley et al., 1994). Antibodies for these experiments include: rabbit polyclonal anti-GFP (a generous gift of Dr J. Fransen, University of Nijmegen, The Netherlands); monoclonal anti-ER-Golgi intermediate compartment 53 (anti-ERGIC53) (kindly provided by Dr H. P. Hauri, University of Basel, Switzerland); rabbit polyclonal anti-NH tag (Elgersma et al., 1997); monoclonal anti-haemagglutinin (HC185) (kindly provided by Dr J. Skehel, NIMR, UK); rabbit polyclonal anti-protein disulfide isomerase (anti-PDI) (kindly provided by Dr I. Braakman, University of Utrecht, The Netherlands); monoclonal anti-Adrenoleukodystrophy protein (anti-ALDP) (a generous gift of Dr P.Aubourg, INSERM, Paris, France); monoclonal anti-catalase (kindly provided by Dr E. Middelkoop, AMC, The Netherlands); rabbit polyclonal anti-oxidase (Furuta et al., 1982). Fluorescently labelled secondary antibodies were obtained from commercial sources (Jackson-ImmunoResearch Laboratories).

Plasmid construction

*Hs*Pex16-EGFP was created by PCR amplification of human liver cDNA using the primers 5′-GGAAGATCTGCCACCATGGAGAA-GCGCGGCTCCTGGGC-3′ and 5′-AAAAGTCGACCCCCAACT-GTAGAAGTAGATTTTC-3′. The resulting PCR product was digested with *Bgl*II and *Sal*I and cloned between the *Bgl*II and *Sal*I sites of pEGFP-N1 (Clonetech). *Hs*Pex3-EGFP was constructed in a similar way using the *PEX3*-specific primers 5′-GAAGATCTGCCACCA-TGCTGAGGTCTGTATGGAATT-3′ and 5′-AAAAGTCGACTTCT-CCAGTTGCTGAGGGGTAC-3′ and EST 128960 as a template. The PCR product was digested with *Bgl*II and *Sal*I and cloned between the *Bgl*II and *Sal*I sites of pEGFP-N1. *Rn*Pex2-EGFP was created by PCR amplification on rat liver cDNA using the primers 5′-CGGGATCCACCATGGCTGCCAGAGAAGAGAG-3′ and 5′-TTT-TCTGCAGAAGAGCATTCACTTCTGACATTT-3′. The PCR product was digested with *Bam*HI and *Pst*I and cloned between the *Bgl*II and *Pst*I sites of pEGFP-N1. Similarly EGFP-*Rn*PMP70 was generated by PCR amplification on rat liver cDNA with the *PMP70*-specific primers 5′-CGGGATCCATGGCGGCCTTCAGC-3′ and 5′-AAAAC-TGCAGCTATGATCCGAACTCAACTG-3′. The PCR product was digested with *Bam*HI and *Pst*I and inserted into EGFP-C1 (Clonetech) cut with *Bam*HI and *Pst*I. All PCR-generated clones were verified by sequencing.
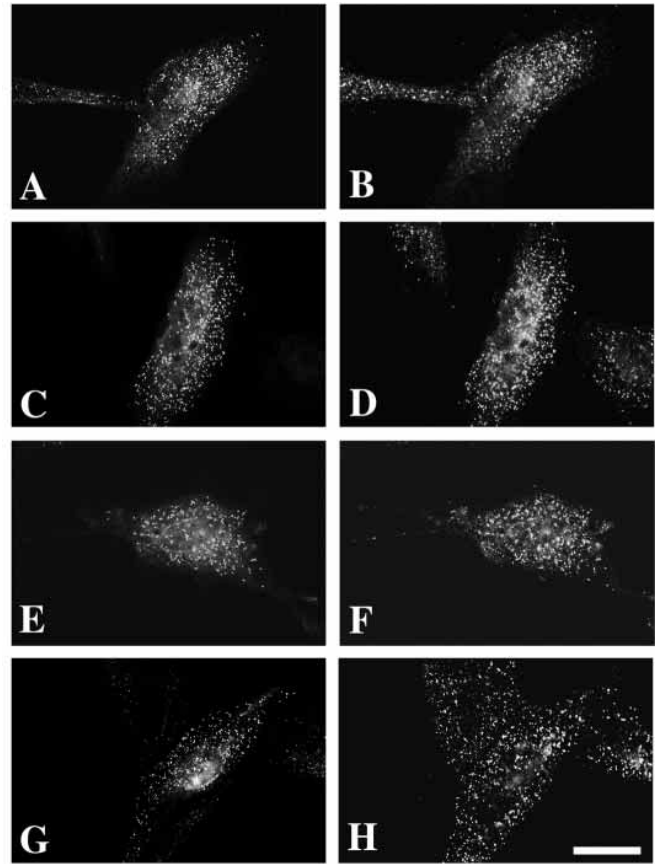
Fig. 1. PMPs are rapidly targeted to peroxisomes. Normal human fibroblasts were microinjected with DNA constructs expressing Pex2p-GFP (A,B), Pex3p-GFP (C,D), Pex16p-GFP (E,F) and GFP-PMP70 (G,H). After 90 minutes, cells were fixed and processed for double indirect immunoflourescence with antibodies to GFP (A,C,E,G) and ALDP (B,D,F,H).

To generate the plasmid expressing influenza virus haemagglutinin (HA), pBD16 (Distel et al., 1998) was digested with *Hin*dIII and *Bam*HI and the fragment encompassing the complete HA protein was cloned between the *Hin*dIII and *Bam*HI sites of pcDNA3 (Invitrogen). The cDNA encoding a GTP-restricted mutant of Sar1p (H79G) in pET-11d HIS (Novagen) was a generous gift of W. E. Balch (Scripps Research Insitute, La Jolla, CA). The His6 tag in the vector was replaced by the NH tag by ligating two complementary oligonucleotides: 5′-CATGGAAGCTTGCCACCATGCAAGACCT-TCCAGGAAATGACAACAGCACAGCAGGTCA-3′ and 5′-TATG-ACCTGCTGTGCTGTTGTCATTTCCTGGAAGGTCTTGCATGGT-GGCAAGCTTTC-3′ between the *Nco*I and *Nde*I sites of pET-11d HIS/Sar1p (H79G). The resulting plasmid was digested with *Hin*dIII and *Bam*HI and the fragment encoding NH-Sar1p (H79G) was gel purified and cloned between the *Hin*dIII and *Bam*HI sites of pcDNA3 (Invitrogen).

RESULTS

Newly synthesized PMPs are first detected in peroxisomes

An ER involvement in peroxisome formation predicts that a subset of PMPs is first targeted to the ER and then transported to peroxisomes, possibly via vesicular transport (Kunau and Erdmann, 1998; Titorenko et al., 2000; Titorenko and

Rachubinski, 1998b). PMPs that have been suggested to travel via the ER are *Y. lipolytica* Pex2p and Pex16p (Titorenko and Rachubinski, 1998a), *Hansenula polymorpha* and human Pex3p (Baerends et al., 1996; Kammerer et al., 1998) and *Saccharomyces cerevisiae* Pex15p (Elgersma et al., 1997). For three of these PMPs, Pex2p, Pex3p and Pex16p, the mammalian orthologues have been identified (Ghaedi et al.,

2000; Honsho et al., 1999; Kammerer et al., 1998; South and Gould, 1999; South et al., 2000; Tsukamoto et al., 1991). We constructed GFP fusions for these three PMPs as well as for a fourth PMP, PMP70, which was previously shown to be directly inserted from the cytosol into the peroxisome membrane (Imanaka et al., 1996). The genes encoding these fusion proteins were cloned into expression vectors and the DNA constructs were microinjected into normal human fibroblasts. At different time points after injection, we examined the distribution of the GFP-tagged PMPs by indirect immunofluorescence using antibodies specific for GFP. Within 90 minutes after injection, expression of the tagged PMPs could be detected and gave rise to a typical punctate peroxisomal pattern of labelling (Fig. 1). At this time point, all tagged PMPs colocalized with the endogenous PMP marker, ALDP (Mosser et al., 1994). At no time point after injection was a different pattern of labelling seen. These results indicate that GFP tagging does not interfere with proper targeting of these PMPs to peroxisomes and that, at the earliest time point of detection (90 minutes after injection), they are localized to peroxisomes only and not to any other cellular compartment.

Peroxisomal targeting of newly synthesized PMPs does not require COPI-mediated vesicular transport

If PMPs are only transiently associated with the ER and then rapidly transported to peroxisomes, an ER localization would go undetected in our microinjection experiments. Therefore, we next tested whether inhibition of COPI-mediated vesicular transport could interfere with targeting of newly synthesized PMPs to peroxisomes. To inhibit COPI assembly, we preincubated normal human fibroblast for 20 minutes with BFA and then microinjected the plasmids expressing GFP-tagged PMPs. As a control, cells were also injected with a plasmid expressing HA, a protein that enters the ER and follows the vesicle-mediated transport pathway to the cell surface (Wiley and Skehel, 1987). The cells were maintained in BFA and processed for double indirect immunofluorescence at various times after injection. GFP-tagged PMPs could be detected in peroxisomes within 90 minutes of injection, as shown by their colocalization with ALDP (Fig. 2A-H). These results indicated that BFA did not inhibit sorting of newly synthesized PMPs to peroxisomes. Moreover, we failed to observe, at any time point after injection, colocalization of GFP-tagged PMPs with marker proteins of other cellular compartments such as ERGIC53 (ER-Golgi intermediate compartment), giantin (Golgi) or PDI (ER) (data not shown). By contrast, HA accumulated in an ER-like pattern and colocalized with PDI (Fig. 2I,J) in the presence of BFA but was efficiently transported to the plasma membrane in untreated cells (Fig. 2K,L).

Previous studies have reported changes in peroxisome morphology in a temperature sensitive CHO mutant containing a mutation in the ε subunit of COPI (Passreiter et al., 1998). Therefore, we analysed the morphology of peroxisomes in normal human fibroblasts treated with BFA. Cells were fixed at various times after the start of the incubation and processed for indirect immunofluorescence using antibodies specific for a peroxisomal matrix protein, acyl-CoA-oxidase, and a Golgi marker protein, giantin. The peroxisome morphology of cells treated for 20 hours with BFA was indistinguishable from untreated cells (Fig. 3A,C). The Golgi-resident protein giantin
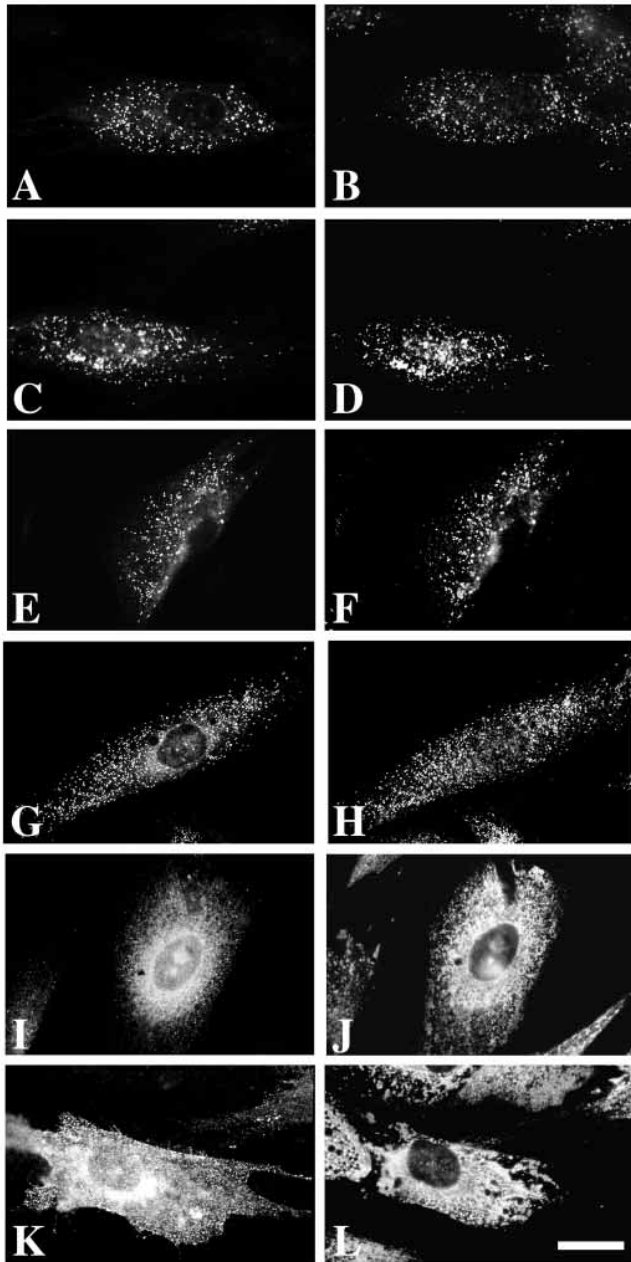
Fig. 2. Peroxisomal targeting of PMPs is not inhibited by BFA. Normal human fibroblasts were incubated in either the presence (A-J) or the absence (K,L) of 2 µg ml^{-1} BFA for 20 minutes and then microinjected with DNA constructs expressing Pex2p-GFP (A,B), Pex3p-GFP (C,D), Pex16p-GFP (E,F), GFP-PMP70 (G,H) and HA (I-L). After microinjection, the cells were maintained in mock or BFA-containing media for 90 minutes and then processed for double indirect immunofluorescence with antibodies to GFP (A,C,E,G) and ALDP (B,D,F,H), or with antibodies to HA (I,K) and PDI (J,L).
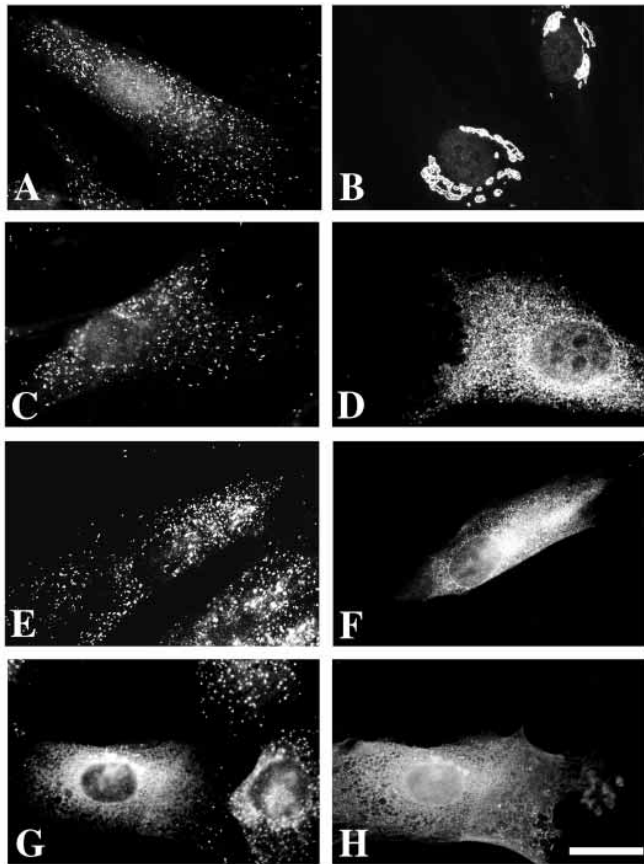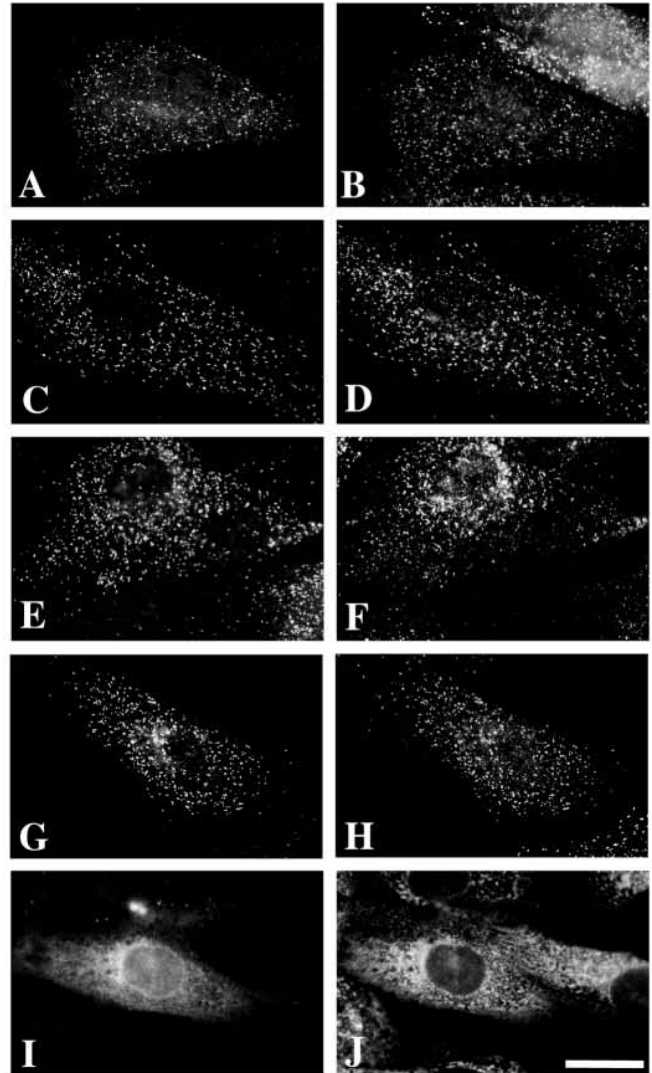
Fig. 3. Inhibitors of COPI and COPII do not affect peroxisome morphology. Normal human fibroblasts were incubated either in the absence (A,B) or in the presence (C,D) of 2 µg ml^{-1} BFA. After 20 hours, cells were fixed and stained with antibodies to acyl-CoA-oxidase (A,C) or giantin (B,D). NH-tagged Sar1p(H79G) DNA was microinjected into the nucleus of normal human fibroblasts. After 20 hours, cells were processed for double indirect immunofluorescence with antibodies to catalase (E) and the NH epitope (F), or with antibodies to ERGIC53 (G) and the NH epitope (H).

Fig. 4. Inhibition of COPII-mediated trafficking does not affect PMP targeting to peroxisomes. Normal human fibroblasts were co-injected with either NH Sar1p(H79G) and constructs expressing PMP-GFP fusions (Pex2p-GFP (A,B), Pex3p-GFP (C,D), Pex16p-GFP (E,F), GFP-PMP70 (G,H)) or NH Sar1p(H79G) and HA (I,J). After 5 hours, cells were fixed and processed for double indirect immunofluorescence with antibodies to GFP (A,C,E,G) and ALDP (B,D,F,H), or with antibodies to HA (I) and PDI (J).

completely redistributed and exhibited a vesicular staining after BFA treatment (Fig. 3B,D), demonstrating that the toxin was effective at the concentrations used and the time-span applied.

Peroxisomal targeting of newly synthesized PMPs does not require COPII-mediated vesicular transport

It is believed that COPI functions only indirectly in ER-to-Golgi trafficking because it mediates the recycling of proteins necessary for COPII-dependent vesicle formation at the ER (Gaynor et al., 1998). This latter process requires Sar1p, a small ER-associated GTPase (Aridor et al., 1995; Barlowe et al., 1994; Kuge et al., 1995). To test the involvement of COPII in PMP sorting directly, we made use of a dominant negative mutant of Sar1p, H79G. This GTP-restricted mutant has previously been shown to inhibit vesicle-mediated protein transport from the ER (Aridor et al., 1995; Shima et al., 1998). Sar1p(H79G) was epitope tagged at its N terminus with the NH tag (Elgersma et al., 1997) and cloned into an expression plasmid. Normal human fibroblasts were co-injected with plasmids expressing NH-Sar1p(H79G) and GFP-tagged PMPs in a ratio of 3:1 at a concentration of 0.2 mg ml^{-1}. Five

hours after injection, the cells were processed for indirect immunofluorescence. This protocol resulted in a high expression of mutant Sar1p in >90% of the cells that expressed GFP-tagged PMPs (data not shown). Indeed, expression of NH-Sar1p(H79G) blocked transport of newly synthesized HA at the ER, as shown by the congruent fluorescence pattern of HA and the ER marker protein PDI (Fig. 4I,J). However, expression of NH-Sar1p(H79G) did not affect sorting of newly synthesized PMPs to peroxisomes because GFP-tagged PMPs appeared in a typical punctate pattern of labelling and colocalized with the peroxisomal marker ALDP (Fig. 4A-H). A different pattern of labelling for GFP-tagged PMPs was never observed, even in cells expressing very high levels of mutant Sar1p.

To investigate whether inhibition of COPII assembly

affected peroxisome morphology, normal human fibroblasts were microinjected with NH-Sar1p(H79G). 20 hours after injection the cells were processed for indirect immunofluorescence using antibodies specific for the NH tag and for catalase, a peroxisomal marker (Fig. 3E,F). No phenotypic change of peroxisomes was observed upon expression of NH-Sar1p(H79G). However, in NH-Sar1p(H79G)-expressing cells, the endogenous ERGIC53 (a protein known to recycle between Golgi and ER (Hauri and Schweizer, 1992)) accumulated in an ER-like pattern and colocalized with the ER marker PDI (data not shown), suggesting that it had recycled to the ER but was unable to exit the compartment (Fig. 3G,H).

DISCUSSION

In this study, we analysed trafficking of PMPs in order to understand how peroxisomes are formed, either from preexisting peroxisomes or from the ER. An ER involvement in peroxisome biogenesis predicts that a subset of PMPs shuttles through the ER en route to the peroxisome (Kunau and Erdmann, 1998; Mullen et al., 1999; Titorenko and Rachubinski, 1998b). This pathway, which might require vesicle budding and fusion events (Titorenko et al., 2000), would provide not only essential membrane components but also the lipids for the formation of nascent peroxisomes. The PMPs travelling via the ER might then be essential for the early stages of peroxisome biogenesis and cells deficient for these 'early genes' are expected to lack detectable peroxisomes. This phenotype has been observed in cells deficient for either of the PMPs Pex3p or Pex16p (Ghaedi et al., 2000; Hettema et al., 2000; Honsho et al., 1999; South and Gould, 1999; South et al., 2000). Other observations that support an ER connection for these proteins are the accumulation of Pex3p at or in the ER after BFA treatment (Salomons et al., 1997), abberrant ER morphology in cells overexpressing Pex3p (Baerends et al., 1996; Kammerer et al., 1998) and N-linked glycosylation of Pex16p (Titorenko and Rachubinski, 1998a). A third PMP inferred to be transported via the ER to peroxisomes is Pex2p. This protein was shown, like Pex16p, to be modified by N-linked glycosylation in *Y. lipolytica* (Titorenko and Rachubinski, 1998a). To follow the kinetics of transport of Pex2p, Pex3p and Pex16p, we epitope tagged these PMPs and microinjected the DNA constructs into the nucleus of normal human fibroblasts. Using this procedure, we are able to detect newly synthesized PMPs within 90 minutes after injection. At this earliest time point of detection, epitope-tagged PMPs were always found in peroxisomes, as shown by colocalization with an endogenous PMP (Fig. 1).

This type of experiment does not, however, exclude the possibility that PMPs are transiently associated with the ER en route to peroxisomes. To further address this issue, we applied inhibitors that affect COPI- and COPII-dependent vesicle formation in the early secretory pathway. If either of these coat proteins is involved in transport of PMPs to peroxisomes, inhibition of their assembly is predicted to result in accumulation of newly synthesized PMPs in a compartment(s) distinct from peroxisomes. First, we inhibited COPI assembly with the fungal toxin BFA. We failed to detect any effect on the localization of these PMPs, whereas, in a parallel

experiment, the same BFA concentrations fully inhibited transport of HA to the plasma membrane, as revealed by the ER accumulation of this protein (Fig. 2). To test the involvement of COPII in PMP sorting, we overexpressed a dominant-negative mutant of Sar1p. Newly synthesized HA, a protein that requires the early secretory pathway for its transport to the plasma membrane, accumulated in the ER upon overexpression of mutant Sar1p. However, we found no inhibition of transport to peroxisomes in cells overexpressing mutant Sar1p for any of the tested PMPs, nor did we observe accumulation of newly synthesized PMPs in the ER or any other non-peroxisomal compartment.

Our results do not support a role for COPI or COPII in PMP sorting. We have restricted our analysis to those PMPs that have been suggested to follow an ER pathway to peroxisomes (Pex2p, Pex3p and Pex16p). A fourth protein that has been inferred to follow this pathway is Pex15p (Elgersma et al., 1997) but recent analysis of Pex15p transport suggested that the ER localization of this protein is caused by its overexpression (Hettema et al., 2000; Stroobants et al., 1999). Our analysis of a limited number of PMPs does not exclude the possibility that other peroxisomal (membrane) proteins follow a COP-dependent pathway to peroxisomes. This is, however, unlikely because we not only tested the transport of specific PMPs but also demonstrated a normal peroxisome morphology in cells treated with COPI and COPII inhibitors (Fig. 3), a phenotype that is not compatible with a COP-dependent peroxisome formation. Similar findings have recently been reported by South and Gould (1999). These authors showed that peroxisomal transport of Pex16p in human fibroblasts is not inhibited by BFA. While our work was in progress, Gould and co-workers reported the analysis of Pex3p transport in human cells (South et al., 2000). In line with our findings, they demonstrated that inhibitors of COPI and COPII have no effect on Pex3p targeting to peroxisomes and do not affect *PEX3*-mediated peroxisome biogenesis. Our data and those of Gould and coworkers demonstrate that PMP targeting and peroxisome formation does not depend on COPI- and COPII-mediated membrane traffic. These data do not, however, definitively rule out a possible role for the ER in peroxisome biogenesis because not all vesicle-budding and -fusion processes depend on COPI and COPII (Latterich et al., 1995). Recent observations in plants suggest that vesicles can be formed at the ER that are morphologically distinct from COP-coated vesicles (Toyooka et al., 2000). Detailed morphological and biochemical analysis showed that certain vacuolar cysteine proteases are synthesized in the ER, where they are packed into large, 300 nm transport vesicles lacking an apparent coat. Interestingly, these vesicles bypass the Golgi complex and fuse directly with protein-storage vacuoles (Toyooka et al., 2000). The molecular mechanism of the formation of these vesicles at the ER remains to be investigated.

If the ER is not the source of the membrane for new peroxisomes, it is difficult to reconcile the intriguing observation that peroxisomes can be synthesized in mutants that apparently lack peroxisomal structures by expression of the originally defective gene (Ghaedi et al., 2000; Honsho et al., 1999; South and Gould, 1999; South et al., 2000). One model, proposed by South and Gould (1999), involves the conversion of a membranous structure, the preperoxisome, into

a vesicle that is competent to import first PMPs and subsequently matrix proteins, eventually converting it into a mature peroxisome. This model, however, still does not explain what the source of this preperoxisomal structure is. As long as we lack any morphological characteristics or biochemical marker for this hypothetical preperoxisomal vesicle we will rely on yeast genetics to resolve this issue. The isolation of new mutants that are disturbed in the very early steps of peroxisome formation and characterization of the affected genes in these mutants might provide clues to the origin of this proposed preperoxisomal vesicle.

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