Communication

Formation of Peroxisomes from Peroxisomal Ghosts in a Peroxisome-deficient Mammalian Cell Mutant upon Complementation by Protein Microinjection*

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Most mammalian cell strains genetically deficient in peroxisome biogenesis have abnormal membrane structures called ghosts, containing integral peroxisomal membrane protein, PMP70, but lacking the peroxisomal matrix proteins. Upon genetic complementation, these mutants regain the ability of peroxisome biogenesis. It is postulated that, in this process, the ghosts act as the precursors of peroxisomes, but there has been no evidence to support this. In the present study, we investigated this issue by protein microinjection to a mutant Chinese hamster ovary cell line defective of PEX5, encoding a peroxisome-targeting signal receptor. When recombinant Pex5p and green fluorescent protein (GFP) carrying a peroxisome-targeting signal were co-injected into the mutant cells, the GFP fluorescence gathered over time to particulate structures where PMP70 was co-localized. This process was dependent on both Pex5p and the targeting signal, and, most importantly, occurred even in the presence of cycloheximide, a protein synthesis inhibitor. These findings suggest that the ghosts act as acceptors of matrix proteins in the peroxisome recovery process at least in the PEX5 mutant, and support the view that peroxisomes can grow by incorporating newly synthesized matrix proteins.

Peroxisome is a ubiquitous organelle found in a wide variety of eukaryotic cells (1). It is a round or oval-shaped structure bounded by a single membrane, ranging from 0.1 to 1.0-μm in diameter. Peroxisomes commonly contain catalase and at least one enzyme which generates hydrogen peroxide. The major roles of peroxisomes, however, change dynamically depending on the species of organisms as well as the physiological environments: e.g. β-oxidation of very long-chain fatty acids and ether lipid synthesis in mammals; fatty acid β-oxidation in fatty seedlings, whereas photosynthesis in green leaves of plants; and assimilation of carbon sources such as n-alkanes, fatty acids, and methanol in yeasts, depending on the carbon sources given.

Knowledge of peroxisome biogenesis has expanded in recent years. As many as 20 genes named PEX (2) have been shown to be involved in peroxisome assembly processes, based on functional complementation on genetically peroxisome-deficient mutants of mammalian cells and yeasts, as well as homology-based screening of EST data bases (for review, see Ref. 3). Products of these genes seem to support protein translocation and membrane assembly of peroxisomes through complex protein/protein interactions (4–7).

In humans, deficiency of one of the PEX genes causes peroxisome biogenesis disorders (PBDs), such as Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease (8, 9). Patients of Zellweger syndrome lack functional peroxisomes and exhibit severe symptoms in various organs including the central nervous system, and death usually occurs in early childhood. On the other hand, patients of milder forms of PBDs seem to have leaky mutations in the corresponding PEX genes, which can often be characterized by temperature sensitivity in peroxisome assembly (10, 11). Fibroblasts of PBD patients in most cases lack functional peroxisomes but have remnant membrane structures called peroxisomal ghosts (12, 13). A peroxisomal membrane protein, PMP70, is present in these membranes, but peroxisomal matrix proteins such as catalase and β-oxidation enzymes are not contained. Similar peroxisome-related abnormal membrane structures are found in peroxisome-deficient mutants of Chinese hamster ovary (CHO) cells and yeasts (9).

In these mutant cells, peroxisomes are restored upon complementation by respective PEX genes. It is an interesting issue whether the peroxisomes in these events are formed from the preexisting ghosts by taking up the matrix proteins from the cytosol, or are newly formed from other structures. This question is closely linked to the long-standing question of where peroxisomes come from (1): preexisting peroxisomes or other structures such as endoplasmic reticulum? In the present study, we employed a CHO cell mutant (14) deficient in PEX5, a gene encoding the peroxisomal targeting signal-1 (PTS-1) receptor (15–17). This mutant strain has typical peroxisomal ghosts containing PMP70. When green fluorescent protein (GFP) attached with PTS-1 was microinjected into the mutant cells together with recombinant Pex5p, the fluorescence gathered to particulate structures, being colocalized with PMP70, even in the presence of cycloheximide, a protein synthesis inhibitor. Hence, the ghosts serve as precursors of peroxisomes upon functional complementation, at least in the PEX5-defective mutant.

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1 The abbreviations used are: PBD, peroxisome biogenesis disorder; PMP70, 70-kDa peroxisomal membrane protein; CHO, Chinese hamster ovary; PTS-1, peroxisomal targeting signal-1; GFP, green fluorescent protein; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Ni-NTA, nickel-nitritriacetic acid; hPa, hectopascal.
Expression and Purification of Recombinant Proteins—Human PEX5 cDNA (long form) (15) was cloned by reverse transcription-polymerase chain reaction, as described (14). The PEX5-coding sequence was inserted in pQE30 vector (QIAGEN), yielding pQEPEX5L. This vector encodes Pex5p, His6-tagged at the amino terminus. The protein expressed in Escherichia coli was purified with Ni-NTA resin, as recommended by the manufacturer.

As a visible peroxisomal marker protein, we used GFPSKL, a fusion protein of GFP(105) and the carboxyl-terminal 25 residues of rat acyl-CoA oxidase (18). The latter sequence contains a Ser-Lys-Leu-COOH tripeptide motif, a typical PTS-1, at the carboxyl terminus (19, 20). As a visible peroxisomal marker protein, we used GFPSKL, a fusion protein of GFP(105) and the carboxyl-terminal 25 residues of rat acyl-CoA oxidase (18). The latter sequence contains a Ser-Lys-Leu-COOH tripeptide motif, a typical PTS-1, at the carboxyl terminus (19, 20).

Microinjection—A PEX5-deficient CHO cell mutant ZP102 (14) was used throughout the study. The mutant cells or wild-type CHO cells (1 × 10⁵/dish) were seeded in 35-mm tissue culture dishes with a glass bottomed microwell (Met Tek, No. 1.5). Each well was coated with 5 µl of mouse type IV collagen solution (0.5 mg/ml) and dried before use. The samples were kept overnight in PBS at 4 °C to remove the nonspecifically bound antibody as much as possible. The samples were further washed five times, 5 min each time, the samples were mounted in 90% glycerol/PBS containing 25 mg/ml of an antibleaching reagent, 1,4-diazabicyclo-(2,2,2)octane, by successive changes of mounting solutions containing increasing concentrations of glycerol.

For fluorescence microscopic observation, an Olympus IX-70 inverted microscope was used. For counting the numbers of peroxisomal ghosts and peroxisomes, the images recorded on photofilms were read with a film scanner, and analyzed by a software package, NIHImage.

RESULTS

Purification of Recombinant Proteins—Human Pex5p (long form), GFP(105), and its SKL-derivative (GFPSKL) were expressed in E. coli as His₆-tagged proteins and purified by Ni-NTA-agarose column chromatography. The purified preparation of Pex5p exhibited a major band of approximately 80 kDa on SDS-PAGE (Fig. 1A), being consistent with a previous report (29). Minor bands were seen in the lower molecular weight region. These bands appeared even when protease inhibitors were added during the disruption of bacteria. This seemed to be because of degradation products generated by endogenous proteases in the bacterial cells, or metal-chelating bacterial proteins. GFP(105) and GFPSKL gave bands of expected sizes on SDS-PAGE (Fig. 1B), with minor contaminants. Some proteolytic cleavage was apparent for GFPSKL.

The functional integrity of recombinant GFPSKL was assessed by microinjection to wild-type CHO cells. Within 24 h
The injected GFPSKL exhibited particulate green fluorescence which was localized to peroxisomes, as revealed by colocalization with the peroxisomal marker, PMP70 (Figs. 2, A and B), whereas GFP(105) itself was not imported to peroxisomes (Fig. 2C). Thus, GFPSKL was translocated to peroxisomes in a signal-dependent manner.

**Rescue of Peroxisomal Import of GFPSKL in the Peroxisome-deficient Cells**—As described previously (14), the PEX5-deficient CHO mutant ZP102 lacks functional peroxisomes. When GFPSKL was injected into ZP102 cells, the GFP fluorescence diffusely distributed throughout the cells (Fig. 2D), being consistent with the inability of this mutant to import peroxisomal matrix proteins. ZP102, however, contained peroxisomal ghosts that were stainable with anti-PMP70 antibody (Fig. 2E). The ghosts were less in number and less sharply stained than peroxisomes of normal CHO cells. When ZP102 cells were injected with both GFPSKL and Pex5p, punctate fluorescence of GFP was observed within 24 h, being superimposable with the PMP70 staining (Fig. 2F and G). If GFP(105) was used instead of GFPSKL, no punctate fluorescence was observed (data not shown). Thus, upon microinjection of the recombinant Pex5p, the mutant cells were complemented with the ability to translocate PTS-1 proteins.

**The Rescue Process Can Proceed in the Presence of Cycloheximide**—Next we questioned whether GFPSKL is directly targeted to the ghosts or is transported there via an indirect pathway in the complementation process of ZP102 cells. To answer this, we carried out the experiment in the presence of a protein synthesis inhibitor, cycloheximide, where ghosts were not expected to be newly formed. GFPSKL exhibited a particulate distribution when Pex5p was co-injected, even though cycloheximide (10 µg/ml) was added (Fig. 2H), and this pattern well accorded with that of PMP70 (Fig. 2I). This import process was through recognition of PTS-1 by Pex5p because punctate fluorescence was not observed when BSA was injected in place of Pex5p (Fig. 2J) or GFP(105) instead of GFPSKL (Fig. 2K). Translocation of GFP-SKL to peroxisomes also occurred in the wild-type CHO cells at indistinguishable efficiencies in either the absence or presence of cycloheximide (data not shown).

To assess the effectiveness of cycloheximide, we compared the cellular number of PMP70-positive particles in the presence and absence of 10 µg/ml cycloheximide, both in the wild-type as well as the mutant CHO cells. We first examined the effect of cycloheximide on cell growth (Table I). Both CHO and ZP102 cells proliferated over 2.5-fold within 24 h, and cycloheximide blocked the growth completely for both strains. The number of ghosts or peroxisomes per cell was nearly constant before and after 24 h either in the presence or absence of cycloheximide, though the average number of PMP70-positive particles per cell was significantly larger in the wild-type CHO than in ZP102. A lower abundance of ghosts was also reported in the fibroblasts from human PBD patients, as compared with that of peroxisomes in normal subjects (24). Thus, net increase in the number of ghosts or peroxisomes, as represented by their number per dish, did not occur in the presence of cycloheximide, whereas the number increased in proportion to the increase in cell number in the absence of cycloheximide. We also observed that the above concentration of cycloheximide suppressed the incorporation of radioactive methionine into tri-chloroacetic acid-precipitable materials by 98% during 5 h of incubation, in both wild-type CHO and ZP102. These findings indicate that cycloheximide of the concentration used in the present study was fully effective in inhibiting de novo protein synthesis, and most importantly, de novo formation of peroxisomes or ghosts.

**Time Course of Peroxisome Recovery**—The peroxisome recovery process in ZP102 upon microinjection of Pex5p was followed in living cells (Fig. 3). Particulate distribution of GFPSKL appeared as early as 1 h after injection and proceeded with time. Cytosolic fluorescence of GFPSKL could still be observed the injected GFPSKL exhibited particulate green fluorescence which was localized to peroxisomes, as revealed by colocalization with the peroxisomal marker, PMP70 (Fig. 2, A and B), whereas GFP(105) itself was not imported to peroxisomes (Fig. 2C). Thus, GFPSKL was translocated to peroxisomes in a signal-dependent manner.

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5 h after injection, whereas incorporation into peroxisomes was almost completed by 24 h (compare Figs. 2 and 3). Thus, the translocation of GFP-SKL to the ghosts (or recovering peroxisomes) continued over many hours.

**DISCUSSION**

The deficiency in the import of a PTS-1 protein to peroxisomes in the PEX5-defective mutant, ZP102 was complemented by injecting a normal PEX5 gene product. This process was not blocked by the protein synthesis inhibitor, cycloheximide, which effectively blocked de novo protein synthesis and formation of PMP70-positive particles. Therefore, at least in the PEX5 mutant, the ghosts are ready to import the peroxisomal matrix proteins from the cytosol, simply upon the supply of Pex5p. Thus, from a functional point of view, the ghosts are empty peroxisomes, accommodating all the necessary sets of components for taking up peroxisome matrix proteins, other than Pex5p.

Our results have implications regarding the question of the intracellular origin of peroxisomes. For many years, peroxisomes have been thought to be derived from preexisting peroxisomes (25). They may be enlarged by importing newly synthesized peroxisomal proteins directly from the cytosol and may proliferate by fission. Recent studies, however, suggest the involvement of endoplasmic reticulum in peroxisome assembly, at least for some integral membrane components (26). Certain yeast Pex proteins accumulate in endoplasmic reticulum when overexpressed (27, 28), one of which is glycosylated (28). In a yeast species, the proteins involved in the secretory pathways and peroxisome assembly seem to be functionally overlapping (29). Moreover, the ADP-riboseylation factor and coatomers known to be important in membrane vesicle formation also seem to be involved in peroxisome biogenesis (30). The functions of these factors were discussed in terms of both vesicle formation from the endoplasmic reticulum and the fission of preexisting peroxisomes (30). Thus, it is again a current question as to whether peroxisomes are formed from preexisting peroxisomes or endoplasmic reticulum.

In the peroxisome recovery process in the PEX5 mutant, net protein transfer from endoplasmic reticulum is probably not required because de novo synthesis of such vesicular components is not likely to occur in the presence of cycloheximide. Most feasible is the interpretation that the ghosts take up the cytosolic GFP-SKL directly, not involving intermediate structures, though possible involvement of recycling transport vesicles should not be ignored. We also observed that peroxisomal translocation of GFP-SKL occurs in normal CHO cells in the presence of cycloheximide, in accordance with a previous report (31). Thus, in normal cells, mature peroxisomes that are equipped with a complete set of Pex machineries would incorporate matrix proteins from the cytosol and may proliferate through division, as suggested (32).

Our results, however, do not necessarily conflict with the possible involvement of vesicular transport derived from the endoplasmic reticulum or other structures. Indeed, peroxisome-deficient cells of several complementation groups do not contain cytologically detectable ghosts (9, 32–36), but nevertheless they regain peroxisome biogenesis activity upon genetic complementation. Certain precursor membranes must serve for the restoration process in these mutant cells, though their entities are not clear. The two mechanisms, fission of preexisting peroxisomes and transfer of membrane components, may be compatible to each other, as has been discussed (32).

**REFERENCES**

1. Lazaro, P. B., Robbi, M., Fujiki, Y., and Wong, L. (1982) *Ann. N. Y. Acad. Sci.* **386**, 285–300.

2. Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dodd, G., Fujiki, Y., Goodman, J. M., Just, W. W., Kiel, J., Kunau, W. H., Lazaro, P. B., Manns aer, G. P., Moser, H. W., Osuni, T., Rachubinski, R., A., Roscher, A., Subramani, S., Tabak, H. F., Tsukamoto, T., Valle, D., van der Klei, I., and van Veldhoven, P. P. (1996) *J. Cell Biol.* **135**, 1–3.

3. Erdmann, R., Veenhuis, M., and Kunau, W. H. (1997) *Trends Cell Biol.*, 400–407.

4. Dodd, G., and Gould, S. J. (1996) *J. Cell Biol.* **135**, 1763–1774.

5. Albertini, M., Rehling, P., Erdmann, R., Girzalsky, W., Kiel, J., Kunau, V., and S., W., van der Heide, M, Faber, 10. Imamura, A., Tsukamoto, T., Osumi, T., and Fujiki, Y. (1998) *Hum. Mol. Genet.* **7**, 2098–2094.

6. Santos, M. J., Inoue, T., Shio, H., and Lazarow, P. B. (1988) *J. Biol. Chem.* **263**, 10992–10999.

7. Santos, M. J., Inoue, T., Shio, H., Small, G. M., and Lazarow, P. B. (1988) *Science* **239**, 1536–1538.

8. Tsukamoto, T., Boga, A., Okumoto, K., Tateishi, K., Fujiki, Y., Shimozawa, N., Suzuki, Y., Kiel, J., Osumi, T., and Fujiki, Y. (1997) *Biochim. Biophys. Res. Commun.* **230**, 402–406.

9. Dodi, G., Braverman, N., Wong, C., Moser, H. W., Watkins, K., Valle, D., and Gould, S. J. (1995) *Nat. Genet.* **9**, 115–125.

10. Fransen, M., Brees, C., Baumgart, E., Vanhooren, J., Baes, M., Manns aer, G. P., and van, Veldhoven, P. P. (1988) *J. Biol. Chem.* **270**, 7731–7736.

11. Wiers, R. N., Nuttley, M. H., Bertolaet, B. L., Li, X., Francke, U., Wheelock, M. J., Anné, U. K., Johnson, K., R., and Subramani, S. M. (1995) *J. Cell Biol.* **130**, 51–65.

12. Miyazawa, S., Hayashi, H., Hikita, M., Ibei, N., Furuta, S., Kagamiyama, H., Osumi, T., and Hashimoto, T. (1987) *J. Biol. Chem.* **262**, 8131–8137.

13. Miyazawa, S., Osumi, T., Hashimoto, T., Ohno, K., Miura, S., and Fujiki, Y. (1989) *Mol. Cell. Biol.* **9**, 83–91.

14. Miura, S., Kasuya-Arai, I., Mori, H., Miyazawa, S., Osumi, T., Hashimoto, T., and Fujiki, Y. (1992) *J. Biol. Chem.* **267**, 14405–14411.

15. Yamakami, M., Hashiguchi, N., Tsukamoto, T., and Osumi, T. (1998) *Bioimages* **2**, 1–7.

16. Tsukamoto, T., Miura, S., Nakai, T., Yokota, S., Shimozawa, N., Suzuki, Y., Orii, T., Fujiki, Y., Osuni, T., and Fujiki, Y. (1998) *Annu. Rev. Cell Biol.* **16**, 245–273.

17. Chaff, C. C., South, S., Warren, D., Jones, J., Moser, A. B., Moser, H. W., and Gould, S. J. (1999) *J. Cell Sci.* **112**, 1579–1590.

18. Lazaro, P. B., and Fujiki, Y. (1985) *Annu. Rev. Cell Biol.* **1**, 489–530.

19. Kunau, W. H., and Erdmann, R. (1997) *Biochimica et Biophysica Acta* **132**, 7326–7341.

20. Titterton, V. I., Oryudziak, D. M., and Rachubinski, R. A. (1997) *Mol. Cell. Biol.* **17**, 5210–5226.

21. Passecker, M., Anton, M., Lay, D., Frank, R., Harter, C., Wieland, F. T., Gorgas, K., and Just, W. W. (1998) *J. Biol. Chem.* **273**, 4169–4176.

22. Hill, P. E., and Walton, P. A. (1998) *J. Biol. Chem.* **273**, 1469–1476.

23. South, S. T., and Gould, S. J. (1999) *J. Biol. Chem.* **274**, 1455–1466.

24. Honsho, M., Tsumoto, S., Shimozawa, N., Suzuki, Y., Orii, T., Fujiki, Y., and Kondo, N. (1999) *Hum. Mol. Genet.* **8**, 1077–1083.