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Phosphoinositide synthesis and degradation in isolated rat liver peroxisomes

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Abstract **Analyzing peroxisomal phosphoinositide (PId[#]) synthesis in highly purified rat liver peroxisomes we found synthesis of phosphatidylinositol 4-phosphate (PtdIns4*P*), PtdIns(4,5)P_2 and PtdIns(3,5)P_2. PtdIns3*P* was hardly detected in vitro, however, was observed in vivo after [^{32}P]-phosphate labeling of primary rat hepatocytes. In comparison with other subcellular organelles peroxisomes revealed a unique PId pattern suggesting peroxisomal specificity of the observed synthesis. Use of phosphatase inhibitors enhanced the amount of PtdIns4*P*. The results obtained provide evidence that isolated rat liver peroxisomes synthesize PIds and suggest the association of PId 4-kinase and PId 5-kinase and PId 4-phosphatase activities with the peroxisomal membrane.**

Keywords: Peroxisomes; Phosphoinositides; Phosphatidylinositol kinases; Phosphatidylinositol phosphatases; Phosphoinositide monophosphate; Phosphoinositide bisphosphate

1. Introduction

Recently published data provide evidence that in *Saccharomyces cerevisiae* Pex3p, an early peroxin, inserts into the membrane of the endoplasmic reticulum (ER) and transport to peroxisomes requires its interaction with Pex19p [1]. In mouse dendritic cells an ER-derived structure containing Pex13p and the 70 kDa peroxisomal membrane protein (PMP) was proposed to be involved in the biogenesis of mammalian peroxisomes [2]. While these observations suggest the ER to play a dominant role in the formation of new peroxisomes, studies on the expression of Pex11αp and Pex11βp in mammalian cells indicated that during peroxisome proliferation new peroxisomes might develop from pre-existing ones [3]. The involvement of the ER raises many questions as for example to the way of transport, the implication of coat proteins, the target membranes for vesicle fusion or the existence of an ER-peroxisome shuttle. Recently, a model on peroxisome proliferation has been proposed based on the observation of multiply constricted peroxisomes implying formation of peroxisomal membrane clusters accumulating distinct PMPs, constriction of tubular peroxisomes and final fission of peroxisomal vesicles [4,5]. Thus, formation of ER-derived and peroxisome-derived peroxisomes might represent multistep processes implying a number of components, such as the small GTPases ARF and RhoA and the dynamin-like protein 1 (DLP1) to peroxisomes [6–8]. Whereas DLP1 might be implicated in a late step of peroxisome proliferation, RhoA was proposed to mediate actin assembly on peroxisomes [9]. ARF and coatomer recruitment were first identified on Golgi membranes and the molecular mechanisms leading to the formation of COP I-coated vesicles were elucidated [10,11]. The process serves the vesicular transport of cargo molecules both in anterograde and retrograde direction [12]. In addition, ARF was shown to be involved in the recruitment of the clathrin coat [13], the control of phospholipase D (PLD) [14] and the membrane association of phosphoinositide (PId)-specific kinases to the Golgi complex [15].

The GTP-dependent recruitment of ARF/coatomer to peroxisomes [16] suggested ARF-GAPs and ARF-GEFs being implicated in ARF activation. Its insensitivity to brefeldin A (BFA) suggested a member of the low molecular weight ARF-GEFs to be involved [17]. As these ARF-GEFs within their common domain structure contain a pleckstrin homology (PH) domain mediating PIds binding, we investigated peroxisomal PId synthesis. We found that isolated peroxisomes in the presence of [γ-^{32}P]ATP synthesize a unique pattern of peroxisomal PIds consisting of PtdIns4*P*, PtdIns(3,5)P_2 and PtdIns(4,5)P_2. Whereas PtdIns4*P* synthesis was predominantly observed in gradient fractions of rat liver homogenate that were rich in endosomal/lysosomal activities, PIP_2 synthesis was maximal in peroxisomes.

2. Materials and methods

2.1. [^{32}P]-Phosphate labeling of primary rat hepatocytes

Highly purified peroxisomes, ER and mitochondria were isolated as previously described [18]. Primary rat hepatocytes were prepared [19] and plated in 10 cm dishes at 3.7×10^6 cells/dish. Cells (20 dishes) were

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Abbreviations: ARF, ADP ribosylation factor; PtdIns, phosphatidylinositol; PIP, phosphoinositide monophosphate; PIP_2, phosphoinositide bisphosphate; PIP_3, phosphoinositide trisphosphate; PId, phosphoinositide; PtdIns4*P*, phosphatidylinositol(4)-phosphate; PtdIns(3,5)P_2, phosphatidylinositol(3,5)-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol(4,5)-bisphosphate; PtdOH, phosphatidic acid; PMP, peroxisomal membrane protein; TLC, thin layer chromatography

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labeled over night with 250 μCi of [^{32}P]-orthophosphate (10 mCi/ml, Amersham Biosciences, Freiburg, Germany) per dish in a phosphate free medium. The post-nuclear supernatant (PNS) was loaded on top of a 14–45% (w/v) Nycodenz gradient and centrifuged in a TV865 rotor at 30000 rpm for 1 h [18]. Labeled phospholipids were extracted, deacylated and analyzed by high pressure liquid chromatography (HPLC, see below). Protein, phospholipid phosphate and the activities of acid phosphatase and 5′-nucleotidase were determined as described [18,20,21].

2.2. Preparation of [^{32}P]-labeled PId standards

Radioactive PtdIns3*P*, PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3 standards were prepared by phosphorylating either phosphatidylinositol (PtdIns), PtdIns4*P*, PtdIns5*P* or PtdIns(4,5)P_2 integrated into phosphatidylserine-based liposomes with human recombinant PId 3-kinase (Alexis Biochemicals, Grünberg, Germany) in the presence of [γ-^{32}P]ATP. [^{32}P]PtdIns4*P* was synthesized by labeling PtdIns-containing liposomes with PId 4-kinase β immunoprecipitated from rat liver cytosol as described [22].

2.3. Incubations and radioactive labeling of cell organelles

Peroxisomes (10 nmol phospholipid phosphate corresponding to 50 μg of protein) were resuspended in 20 μl labeling buffer (25 mM HEPES, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT) and incubated in a total volume of 100 μl containing 4 μCi [γ-^{32}P]ATP (400 μCi/μmol) for varying times at 37 °C. Phosphatase inhibitors were used in a 1:100 dilution of Phosphatase Inhibitor Cocktail 2 (Sigma–Aldrich Chemie, Munich, Germany). Reactions were stopped by the addition of 100 μl 1 M HCl and phospholipids extracted using 400 μl chloroform:methanol (1:1). The organic layer was washed once using 400 μl chloroform:1 M HCl (1:1) and after drying the organic phase under a stream of N_2 the extracted lipids were dissolved in chloroform:methanol (1:1). For labeling of rough ER microsomes and mitochondria we followed exactly the same procedure as used for peroxisomes. In some experiments prelabeled peroxisomes were reisolated and further incubated in labeling buffer in the presence or absence of phosphatase inhibitors.

2.4. Chromatographic separations

Thin layer chromatography (TLC) was performed as described in [23]. The labeled substances were detected by phosphoimaging (Bio-Rad, Munich, Germany) and quantified. For HPLC labeled PIds (250 μCi [γ-^{32}P]ATP, 1.25 mCi/μmol) were obtained from peroxisomes (200 nmol phospholipid phosphate), extracted and the dried lipids deacylated with approximately 50% recovery [24] . HPLC analysis (Merck-Hitachi LaChrom Elite system, VWR, Darmstadt, Germany, equipped with a radioactivity detector, Raytest, Straubenhardt, Germany) was done as described [25] using a Partisphere SAX 5 μm Whatman column 4.6 × 250 mm (Fisher Scientific, Schwerte, Germany) and a 0–250 mM ammonium phosphate buffer gradient of pH 3.8 over 60 min and at a flow rate of 1 ml/min.

2.5. Antibodies

For immunoblotting antibodies were used directed against PMP70p, Pex11αp (peroxisomal membrane proteins) [26], calnexin (an ER-localized chaperone, Stressgen, Biomol, Germany), the F_1-ATPase (mitochondrial inner membrane protein, Santa Cruz Biotechnology, Heidelberg, Germany), EEA1 (endosomal marker protein, BD Biosciences, Heidelberg, Germany). The anti-PtdIns 4-kinase β antibody was from Biomol (Hamburg, Germany) and the anti-rabbit peroxidase-labeled secondary antibodies were from (Dianova, Hamburg, Germany).

3. Results

3.1. Synthesis and identification of peroxisomal PIds

To investigate peroxisomal PId synthesis, we used highly purified peroxisomes from rat liver. Based on marker enzyme activities the peroxisome fraction isolated from clofibrate-treated rat livers was >95% pure. The high purity of this preparation was achieved at the expense of organelle yield. The isolated peroxisome fraction represented about 20% of the total hepatic population. The major contamination in this fraction was derived from ER microsomes [18]. Mitochondrial, endosomal, Golgi and plasma membrane markers were low or even not detected [16]. This peroxisomal preparation was used in the experiments described in Figs. 1, 3, 5 and 6. In Fig. 1A we show the phospholipids extracted from these peroxisomes after [γ-^{32}P]ATP-labeling. Phosphoimaging of the labeled lipids following their TLC separation revealed five radioactive spots. Four of them, phosphatidic acid (PtdOH), PtdIns, phosphatidylinositol monophosphate (PIP), and phosphatidylinositol bisphosphate (PIP_2) showed identical chromatographic behavior as the corresponding phospholipid standards. The substance migrating between PIP and PIP_2, designated PX, has not been identified yet.

To further characterize the synthesized PId species, the lipids extracted after [γ-^{32}P]ATP-labeling were deacylated and the deacylation products subjected to HPLC. The results obtained are shown in Fig. 1B. By comparing the elution profiles of per-

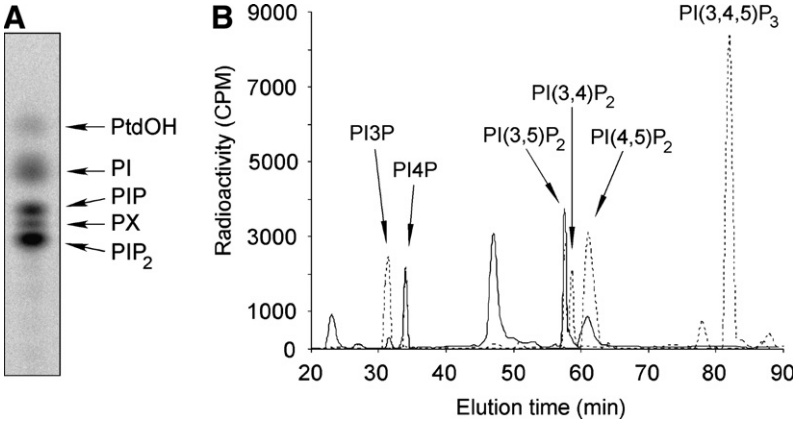

Fig. 1. In vitro synthesis and characterization of peroxisomal PIds. (A) Representative pattern of peroxisomal PIds following [γ-^{32}P]ATP-labeling of highly purified rat liver peroxisomes (10 nmol phospholipid phosphate). After labeling for 30 min the phospholipids were extracted, separated by TLC (1% oxalate-pretreated silica gel plates; 1-propanol:2 M acetic acid 65:35) and the radioactive spots visualized by phosphoimaging. (B) Identification of [γ-^{32}P]-labeled peroxisomal PIds (solid line) by HPLC after extraction and deacylation of the labeled phospholipids. Standard PIds (broken line) used for the identification were prepared as described in Section 2. The PIds identified in peroxisomes included PtdIns(4)*P*, PtdIns(4,5)P_2 and PtdIns(3,5)P_2. The substance eluting at a retention time of 47 min was no known PId and not identified so far.