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Import of phosphatidylserine to and export of phosphatidylethanolamine molecular species from mitochondria

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ABSTRACT

Heavy isotope-labeled ethanolamine and serine as well as exogenous PE and PS species were used to study trafficking of phosphatidylethanolamine (PE) and -serine (PS) molecular species between the endoplasmic reticulum (ER) and mitochondria in HeLa cells. Import of both endogenous and exogenous PS to IMM was a relatively slow process (T1/2 = several hours), but depended on the acyl chains. In particular, the 38:4 and 38:5 species were imported more efficiently compared to the other PS species. Knock-down of Mitofusin 2 or Mitostatin had no detectable effect on PS import to mitochondria, suggesting that the ER–mitochondria contacts regulated by these proteins are not essential. Knock-down of PS synthase 1 inhibited PS decarboxylation, suggesting that import of PS to mitochondria is coupled to its synthesis. Also the export of PE from IMM to microsomes is a relatively slow process, but again depends markedly on the acyl chain structure. Most notably, the polyunsaturated 38:4 and 38:5 PE species were less efficiently exported, which together with rapid import of the PS precursors most probably explains their enrichment in IMM. PE synthesized *via* the CDP-ethanolamine was also imported to IMM, but most of the PE in this membrane derives from imported PS. In contrast to PS, all PC species made in Golgi/ER translocated similarly and rapidly to IMM. In conclusion, selective translocation of PS species and PS-derived PE species between ER and mitochondria plays a major role in phospholipid homeostasis of these organelles.

1. Introduction

Aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) together present approx. 30 mol% of total phospholipids in mammalian cells and are essential as no mutants lacking either phospholipid have been identified [1]. The metabolism of these lipids is tightly linked since PS serves as a major precursor of PE and PS can be synthesized from PE [2,3]. Beside the PS decarboxylation pathway, a significant part of cellular PE is derived *via* the CDP-ethanolamine (CDP-EA), or Kennedy, pathway. The relative contributions of these two pathways to the cellular PE pool are not clear and may vary depending on cell type and culture conditions [4]. Little is also known of the regulation and coordination of these pathways.

The synthesis of PE *via* the decarboxylation pathway is a particularly intriguing process as it requires translocation of PS from the endoplasmic reticulum (ER) to mitochondria where PS is decarboxylated by the phosphatidylserine decarboxylase (PSD) located in the inner mitochondrial membrane (IMM) [5]. The importance of this pathway is demonstrated by the fact that PSD knock-out mice die early in the embryonic phase due to mitochondrial dysfunction [6]. Whether this is due to that PE produced *via* the CDP-EA pathway is not adequately transported to mitochondria or that it produces "wrong" molecular species is not clear. Despite the biological importance of PS translocation to mitochondria, the mechanisms involved remain unresolved. There is no evidence for vesicular trafficking to this organelle and it appears that (soluble) phospholipid transfer proteins are not involved [7,8]. Our previous data indicated that PS molecular species do not translocate from ER or the plasma membrane to mitochondria equally, but the rate of translocation is inversely proportional to the molecular hydrophobicity [9–11], suggesting that spontaneous diffusion (or efflux from the donor membrane) is the rate-limiting step in the translocation process [9–11]. However, other investigators have proposed that substrate preference of PSD, rather than differential translocation of PS species to mitochondria, is responsible for differential labeling of PE molecular species from serine [12–14].

The PS synthases PSS1 and PSS2 are enriched in MAM, *i.e.* ER domains associating with mitochondria [2,15], and several investigators have suggested that translocation of PS to mitochondria takes place

Abbreviations: CDP-EA, CDP-ethanolamine; D-PE, heavy isotope-labeled phosphatidylethanolamine; D-PS, heavy isotope-labeled phosphatidylserine; ESI-MS, electrospray ionization mass spectrometry; IMM, inner mitochondrial membrane; LC–MS, liquid chromatography–mass spectrometry; MAM, mitochondria-associated membrane; mβ-CD, methyl-β-cyclodextrin; SRM, selective reaction monitoring; OMM, outer mitochondrial membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PSS, phosphatidylserine synthase; PSD, phosphatidylserine decarboxylase

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via membrane contact sites between MAM and the outer mitochondrial membranes (OMM) [2,16–19]. Specific proteins, such as Mitofusin 2 and Mitostatin appear to regulate the proximity of MAM and OMM [20,21], but there is no direct experimental evidence for a role of those proteins or membrane contact sites in PS transport in mammalian cells.

Since a major fraction of the cellular PE in various cultured cells is derived from PS [16,22–24], much of the PE formed in mitochondria must be exported from this organelle [2,25], possibly *via* putative MAM/OMM contact sites. However, the mechanism of PE export has not been established. It is also not clear whether all PE species translocate from mitochondria equally or if selectivity based on *e.g.* molecular hydrophobicity exist. It has been reported that PE formed *via* the CDP-EA pathway is not imported to mitochondria [2,26]. However, contradictory results have been reported recently for mammalian cells [12] and yeast [27].

Here, we used mass-spectrometry and heavy isotope-labeled phospholipid precursors to determine the kinetics of import of endogenously synthesized or exogenous PS molecular species to IMM as well as the export of the PS-derived molecular species of PE from IMM. IMM rather than intact mitochondria was studied because only the former could be obtained with adequate purity. We find that both PS import to and PE export from IMM depend on the acyl chains, but there is no simple correlation between molecular hydrophobicity and the rate of transfer. The polyunsaturated PS species 36:4, 38:4 and 38:5 (the numbers indicate the number of acyl chain carbons and double bonds, respectively) are rapidly imported to mitochondria, while the PE species derived thereof were slowly exported from mitochondria. This imbalance between import and export most probably explains the enrichment of these polyunsaturated PE species in mitochondria. Knock-down of Mitofusin 2 and/or Mitostatin had no discernible effect on the decarboxylation of PS, indicating that MAM–mitochondria contacts depending on these proteins are not critical for PS import to mitochondria. On the other hand, inhibition of PS synthesis by knocking down PS synthase 1 decreased PS decarboxylation, suggesting that translocation of PS to mitochondria is coupled to/driven by its synthesis. PE species formed in the ER *via* the Kennedy pathway were translocated to IMM, but most of PE in mitochondria/IMM derives from imported PS.

2. Materials and methods

2.1. Lipids and other chemicals

Culture media and reagents were obtained from Invitrogen, cholesterol and other unlabeled lipids from Avanti Polar Lipids (Alabaster, AL), D_3^{15}N-L-serine, D_3-L-serine, D_4-ethanolamine and D_9-choline from CIL (Andover, MA), methyl-β-cyclodextrin (meβ-CD), phospholipase D (*Streptomyces* sp.), and hydroxylamine (HA) from Sigma and methyl arachidonoyl fluorophosphonate (MAFP) was from Cayman Europe. Digitonin was obtained from Calbiochem. All siRNAs were obtained from Qiagen. The siRNAs targeting phosphatidylserine synthase 1 (PSS1) or 2 (PSS2) and AllStars (control) siRNAs (Cat. # SI04169984, SI04255965 and 1027280, respectively) were predesigned, whereas the sequences of siRNAs targeting Mitostatin (TCHP) and Mitofusin 2 (MFN2) were obtained from previous publications [20,21]. QPCR primers for TCHP (Quanti Tect® Primer Assay) were from Qiagen. The primer sequences for other genes (*cf.* Supplemental Table 1) have been published (*cf.* Supplementary Information), or were designed using NCBI Primer-BLAST and synthesized by Oligomer (Helsinki, Finland). Lipofectamine™ RNAiMAX was from Invitrogen, Machery-Nagel NucleoSpin® RNA II Kit from Biotop (Helsinki, Finland) and FastStart Universal SYBR Green Master from Roche. All solvents (HPLC-grade) were from Merck.

The PE and PS species with a heavy isotope-labeled head group were synthesized from corresponding phosphatidylcholine species and D_4-ethanolamine or D_3^{15}N-serine, respectively, using phospholipase D-mediated transphosphatidylation as described previously for unlabeled lipids [28] except that the reaction volume was reduced 5-fold. The labeled phospholipids were purified by normal-phase HPLC [29], their purity was confirmed by mass spectrometry and their concentrations were determined using phosphate analysis [30].

2.2. Preparation of donor vesicles

POPC, cholesterol and heavy isotope-labeled PE or PS (1:2:1; molar ratio) were mixed in chloroform/methanol (9:1, v/v) and the solvent was evaporated under a nitrogen stream followed by high vacuum for 1 h. PBS was added and the sample was probe-sonicated for 3×2 min with 30 s intervals. The sample was centrifuged at 3000 *g* for 5 min to pellet any undispersed lipid and probe particles. The small unilamellar vesicles in the supernatant were used as donors of exogenous phospholipid to cells.

2.3. Labeling of cells

HeLa cells were grown as previously [9,31] to ~80% confluency on cell culture dishes. To introduce exogenous, head-group labeled PS or PE species to cells, donor vesicles, mβ-CD (4 or 6 mM), and MAFP (25 μM) in 10 ml of DMEM with 2% FBS were added and the cells were incubated for 20–60 min at 37 °C. After washing with PBS the cells were chased in DMEM containing 2% FBS for 0–8 h. We have previously shown that the donor vesicles do not bind to cells significantly [31]. To label endogenous PS, PE or PC, D_3-L-serine (300 μg/ml), D_4-ethanolamine (100 μg/ml) or D_9-choline (100 μg/ml) were added in DMEM containing 2% FBS and then incubated for the indicated time. After washing with PBS, the cells were "chased" in DMEM containing 2% FBS and equal amount of unlabeled precursor for 0–24 h. Mass spectrometric analyses showed that with each precursor undetectable amounts of D-label were present in the acyl and glycerol moieties of the respective phospholipid at all time points studied. This is consistent with previous data for another cell line [12].

2.4. Cell fractionation and immunoblotting

After washing with PBS, the cells were detached from two 14 cm dishes with trypsin–EDTA, centrifuged for 3 min at 1500 *g*, washed once with PBS and pelleted. After suspending in 1 ml of sucrose–mannitol–Tris–HCl–EGTA (75–225–30–100 mM, pH 7.4) buffer the cells were homogenized by passing 10 times through a 27 G needle and any intact cells remaining were removed by centrifugation twice for 5 min at 1200 *g*. The mitochondria were pelleted by centrifuging at 10,800 *g* for 5 min, the supernatant containing microsomes was moved to an Eppendorf tube and any remaining mitochondria were removed by centrifugation at 20,800 *g* for 5 min. The mitochondrial pellet was washed twice with 500 μl of sucrose–mannitol–Tris–HCl (75/225/30 mM, pH 7.4) buffer and then suspended in 100 μl of sucrose–mannitol–Tris–HCl–EGTA (75/225/30/100 mM, pH 7.4) buffer and samples were taken for protein determination [32]. To obtain inner mitochondrial membranes (IMM), digitonin in sucrose–mannitol–Hepes (70/200/2 mM, pH 7.4) was added to a suspension of crude mitochondria containing BSA (0.012 mg BSA per mg mitochondrial protein) to obtain a digitonin to mitochondrial protein ratio of 2:1. After 15 min on ice, BSA in sucrose–mannitol–Hepes buffer was added to obtain BSA to mitochondrial protein ratio of 0.24/1 and IMM was pelleted by centrifugation at 12,200 *g* for 5 min.

To estimate purity of the fractions the proteins were separated on a 10% SDS-PAGE gel and transferred to PVDF (Millipore) membranes, which were then blocked for 1 h in TBS containing 5% non-fat milk and 0.1% Tween-20. Antibodies against calnexin (1:10,000, BD Biosciences), LAMP2 (1:20,000, Developmental Studies Hybridoma Bank), VDAC (1:10,000, Calbiochem) and COXII (1:2000, Santa Cruz Biotechnology) were used to detect ER/MAM, lysosomes, OMM or