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Enzyme-coupled assays for flip-flop of acyl-Coenzyme A in liposomes

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ABSTRACT

Acyl-Coenzyme A is made in the cytosol. Certain enzymes using acyl-CoA seem to operate in the lumen of the ER but no corresponding flippases for acyl-CoA or an activated acyl have been described. In order to test the ability of purified candidate flippases to operate the transport of acyl-CoA through lipid bilayers in vitro we developed three enzyme-coupled assays using large unilamellar vesicles (LUVs) obtained by detergent removal. The first assay uses liposomes encapsulating a water-soluble acyl-CoA:glycerol-3-phosphate acyl transferase plus glycerol-3-phosphate (G3P). It measures formation of [^{3}H]lyso-phosphatidic acid inside liposomes after [^{3}H]palmitoyl-CoA has been added from outside. Two other tests use empty liposomes containing [^{3}H]palmitoyl-CoA in the inner membrane leaflet, to which either soluble acyl-CoA:glycerol-3-phosphate acyl transferase plus glycerol-3-phosphate or alkaline phosphatase are added from outside. Here one can follow the appearance of [^{3}H]lyso-phosphatidic acid or of dephosphorylated [^{3}H]acyl-CoA, respectively, both being made outside the liposomes. Although the liposomes may retain small amounts of detergent, all these tests show that palmitoyl-CoA crosses the lipid bilayer only very slowly and that the lipid composition of liposomes barely affects the flip-flop rate. Thus, palmitoyl-CoA cannot cross the membrane spontaneously implying that in vivo some transport mechanism is required.

1. Introduction

Living organisms are surrounded by lipid-containing membranes, which in eukaryotes are mainly made of glycerophospholipids, sphingolipids and sterols. Glycerophospholipids are made from phosphatidic acid (PA), a central metabolite, which in eukaryotes is generated through acyl-Coenzyme A (acyl-CoA) dependent reactions from glycerol-3-phosphate (G3P) [1]. G3P is first acylated in *sn*-1 by a glycerol-3-phosphate acyltransferase (GPAT) and then in *sn*-2 by a 1-acylglycerol-3-phosphate acyltransferase (AGPAT). The numerous eukaryotic GPATs and AGPATs mostly belong to either one or the other of two gene families, characterized by the pfam01553 and pfam3062 motifs, respectively. GPATs and AGPATs found in animals and yeast are transmembrane proteins, while chloroplasts of some plant species contain soluble luminal GPAT homologs, one of which, the one of squash (*Cucurbita moschata*), has been crystallized [2,3]. The pfam3062 motif is characteristic of membrane bound O-acyltransferases (MBOATs), a huge superfamily of ER proteins with multiple transmembrane domains. A strictly conserved His residue in the pfam3062 motif is considered to be the critical active site residue [4] and, in eukaryotes, to reside in the ER lumen. The lumenal location of the active site of MBOAT proteins is not only supported by biochemical investigations of the topology of the conserved His residue [5–9], but also by the fact that some MBOATs acylate ER lumenal secretory proteins such as Hedgehog, Wnt, Ghrelin and yeast GPI anchored proteins [10–14]. Also, lyso-GPI anchors are acylated in the lumen of the mammalian Golgi by PGAP2 [15] and the GPI biosynthetic intermediate phosphatidylinositol-glucosamine (PI-GlcN) is acylated on the inositol moiety in the ER lumen by Gwt1 [16,17]. PGAP2 and Gwt1 do not belong to the abovementioned pfam motif families. Acyl-CoA dependent acylation reactions in the lumen of the secretory apparatus imply that acyl-CoA or the acyl group of acyl-CoA may have to be transported through organellar membranes.

Best characterized are the mechanisms used to bring fatty acids into mitochondria or peroxisomes for β-oxidation. In mitochondria, acyls are transferred from CoA to carnitin and transported as acyl-carnitin through the inner membrane, to then be transferred again onto CoA in the matrix. There also is compelling evidence for the role of two ATP-driven ABC half transporters, Pxa1 and Pxa2 required for the import of long chain fatty acids into yeast peroxisomes and similar transporters exist in mammals and plants. However, it still is not totally clear if Pxa1/2 act as bona fide acyl-CoA flippases or only transport the fatty

Abbreviations: AGPAT, 1-acylglycerol-3-phosphate acyltransferase; AP =, bovine intestinal alkaline phosphatase; BSA, bovine serum albumin; Cho, cholesterol; CoA, Coenzyme A; DOPC, dioleoyl-PC; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LUVs, large unilamellar vesicles; MBOAT, membrane bound O-acyltransferase; OGP, octyl-β-D-glucopyranoside; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PFO, perfringolysin O; POPC, palmitoyl-oleoyl-PC; POPE, palmitoyl-oleoyl-PE; POPS, palmitoyl-oleoyl-PS; PS, phosphatidylserine; RT, room temperature; TX-100, Triton X-100.

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acid moiety [18–20]. With regard to the ER, no acyl-CoA flippases have been identified yet.

Here we undertook to set up assays that may be used to measure the activity of acyl-CoA transporters after their reconstitution into large unilamellar vesicles (LUVs).

2. Materials and methods

Sources of materials can be found in the Supplementary materials.

2.1. Encapsulation of sqGPAT and G3P into large unilamellar vesicles (LUVs)

Liposomes with encapsulated sqGPAT and G3P were formed by detergent removal using Bio-bead SM-2 adsorption [21]. In the standard protocol, 2.5 mg of lipids was dried under vacuum for 4 h at 37 °C. 1 ml of buffer D (20 mM potassium phosphate buffer pH 7.0, 50 mM NaCl) containing 1% of detergent (C12E9, octyl-β-D-glucopyranoside (OGP) or TX-100), 1 mM G3P and 15 nmol (657 μg) sqGPAT was added to the dried lipids; the tube was shaken gently for 30 min on a rotating wheel. Thereafter, detergent was removed using SM-2 beads adapting the protocol of [22]: For C12E9 or OGP, the solution was incubated on an end-over-end rotator with 150 mg (wet weight) of SM-2 beads for 2 h. Then, 300 mg of fresh beads was added and shaking was continued for 12 h (overnight). Thereafter, the liposome solution (without beads) was transferred to a new tube containing 500 mg of fresh SM-2 beads and incubated for an additional 3 h. All steps were performed at 4 °C unless stated otherwise. TX-100 was removed as above but using 150 mg of beads, incubating 3 h, adding 250 mg of fresh beads and incubating another 3 h. Liposomes were then transferred to fresh 300 mg beads for 12 h, and finally to fresh 300 mg beads for 2 h. This procedure results in the formation of LUVs, the size of which depends on the detergent: C12E8 yields vesicle populations with a modal diameter of 90 nm, TX-100 of 240 nm and OGP of >300 nm [23]. Unless indicated otherwise, OGP was used, but the same results were obtained with liposomes made using C12E9 or TX-100.

To digest non-encapsulated sqGPAT potentially sticking to the outside of the LUVs, the liposomes were incubated with 50 μg (1.73 μM) proteinase K at 4 °C for 1 h. Proteinase K was inactivated by adding PMSF and AEBSF to 2 and 0.5 mM and further incubating for 1 h at 4 °C. Inactivated proteinase K and peptides were then removed by dialyzing (cut-off 100 kDa) against 100 ml buffer D containing 1 mM G3P at 4 °C for 15 h. (Dialysis had no effect on the results and was omitted in later preparations.) In all experiments shown here we used proteinase K to shave the liposomes, but trypsin achieved the same result (Fig. S1). Lipid loss was estimated by quantifying phosphatidylcholine (PC) using a choline oxidase based assay [24]. About 15% of initially added PC was lost during liposome preparation. Typically, the final liposome preparations contained about 2.3 mg (3 μmol) of lipid encapsulating 7–17 μg of sqGPAT. The G3P concentration in their lumen was assumed to be 1 mM, as all buffers used throughout contained 1 mM G3P.

2.2. LPA generation in liposomes encapsulating sqGPAT and G3P

In most experiments, [^{3}H]palmitoyl-CoA (1 μCi, 17 pmol) was added to liposomes equivalent to 100 nmol of palmitoyl-oleoyl-PC/cholesterol (POPC/Cho; 70:30 mol%) encapsulating sqGPAT/G3P in a final volume of 100 μl buffer D supplemented with 1 mM G3P. The reaction was incubated up to 60 min at RT. As a positive control, liposomes were preincubated for 10 min at RT with perfringolysin O (PFO) (0.7 nmol) in buffer D supplemented with 1 mM G3P and 1 mM DTT (to maintain PFO activity). Alternatively, detergents (TX-100 or OGP) were added to the assays in a 10-fold molar excess over lipids. Reactions were stopped by the addition of 2.5 μl HCl (1 M) to reach a final pH < 4 (in order to increase solubility of LPA in butanol) and vigorous vortexing. Lipids were extracted 3 times by adding 350 μl butanol to 100 μl of reaction solution (aqueous phase), vortexing and subsequently removing the butanol phase with a pipette. The pooled butanol fractions were dried under vacuum in a rotary evaporator and resuspended in 30 μl of solvent. Samples were applied to TLC plate (silica gel 60) and dried for 60 min under laminar airflow. Samples were analyzed by TLC using the solvent system chloroform/methanol/0.25% KCl (55:45:5). Plates were analyzed by phosphorimaging and radioscanning (Berthold Services GmbH, Switzerland). All quantifications of bands were obtained through radioscanning.

2.3. Incorporation of [^{3}H]palmitoyl-CoA into liposomes

Normally, 2.5 mg of lipids together with [^{3}H]palmitoyl-CoA in organic solvent were mixed and dried under vacuum in the rotary evaporator at 20 °C for 4 h. Buffer D containing 1% of detergent (C12E9, OGP or TX-100) was added and liposomes were prepared as described above. The resulting liposomes containing [^{3}H]palmitoyl-CoA were stored at 4 °C and were usually used within 6 h. Palmitoyl-CoA has detergent-like properties and is also partially removed by the SM-2 beads, but the final liposome preparation usually still contained 5–10% of the radioactivity added in the beginning.

2.4. Phosphatase treatment of liposomes containing [^{3}H]palmitoyl-CoA

Liposomes containing [^{3}H]palmitoyl-CoA were subjected to bovine intestinal alkaline phosphatase (AP) treatment: Typically, 30 μl of [^{3}H]palmitoyl-CoA containing liposomes (100 nmol lipids, 17 pmol [^{3}H]palmitoyl-CoA) was treated with 10 U of AP in a final volume of 100 μl buffer D. In the case of measuring the production of LPA, 60 pmol (2.6 μg) sqGPAT and 100 nmol G3P were added to the liposomes in 100 μl of buffer D. Reactions took place at RT. Reactions were stopped by addition of 2.5 μl HCl (1 M) and lipids extracted and analyzed as described above.

All other methods used are described in the Supplementary materials section.

3. Results

3.1. LUVs can replace bovine serum albumin in the sqGPAT activity assay

As reported before for another soluble GPAT [25], LPA is formed by sqGPAT only in the presence of bovine serum albumin (BSA), which seems to be required for making acyl-CoA more accessible for sqGPAT (Fig. S2A). Yet we found that large unilamellar vesicles (LUVs) could replace BSA in this function if they were added in sufficient quantity (Fig. S2B). As the concentration of palmitoyl-CoA (10 μM) in the assays was below its critical micelle concentration (CMC) of 75 μM [26], data indicate that acyl-CoA monomers are not recognized by sqGPAT, but that the enzyme can utilize acyl-CoA presented by LUVs if the surface density of acyl-CoA falls below a certain critical value, which seems to be around 10–20 mol% (Fig. S2B). On the other hand, sqGPAT did not work in the presence of detergents such as OGP, TX-100, or dodecylmaltoside (tested at 2.5 mM, i.e. above CMC for the last two) (not shown). Also, when added to assays containing BSA, these same detergents inhibited sqGPAT in a concentration dependent manner (not shown).

3.2. Assays using liposomes encapsulating squash GPAT together with G3P

Palmitoyl-CoA can be regarded as a detergent and we wondered at what concentration it would dissolve liposomes. For this we produced LUVs filled with calcein, a fluorophore, which is self-quenching at concentrations of above 50 mM [27]. LUVs made by extrusion, i.e. without detergent, could not be lysed even at palmitoyl-CoA:lipid ratios of 1.6:1, although small transient leakages causing around 1% leakage of total encapsulated calcein were occasionally observed upon addition of acyl-CoA (Fig. S3). However, in view of reconstituting proteins into