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A LYSOPHOSPHATIDIC ACID - BINDING CYTOSOLIC PROTEIN STIMULATES MITOCHONDRIAL GLYCEROPHOSPHATE ACYLTRANSFERASE

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Summary: Rat liver cytosolic fraction caused up to five fold stimulation of mitochondrial glycerophosphate acyltransferase apparently by removing the lysophosphatidic acid formed by the acyltransferase. When mitochondria were incubated with palmityl-CoA, [2-^{3}H]-sn-glycerol 3-phosphate and the cytosolic fraction and the supernatant fluid of the incubated mixture was passed through a Sephadex G-100 column, labeled lysophosphatidic acid eluted in three peaks with M_rs (i) 60-70 kDa, (ii) 10-20 kDa, and (iii) <5 kDa. Proteins, responsible for binding of lysophosphatidic acid in peaks (i) and (ii), were purified to near homogeneity as judged by electrophoretic analysis. The lysophosphatidic acid binding protein in peak (i) appears to be serum albumin and peak (iii) represents largely unbound lysophosphatidic acid. The 15 kDa protein, purified from peak (ii), bound lysophosphatidic acid, stimulated the acyltransferase and export of lysophosphatidic acid from mitochondria.

Glycerophosphate acyltransferase (EC 2.3.1.15, GAT) catalyzes the conversion of sn-glycerol 3-phosphate to lysophosphatidic acid (LPA), the first step in glycerolipid biosynthesis. In mammalian organs, the acyltransferase is located in both the mitochondria and endoplasmic reticulum (microsomes). In the liver, GAT is equally active in both the organelles (1,2). Although both the subcellular fractions esterify fatty acids in position 1 of sn-glycerol 3-phosphate, the mitochondrial acyltransferase exhibits a strong preference for saturated fatty acyl-CoAs (1,2). Therefore, the properties of the mitochondrial, but not the microsomal acyltransferase, are in keeping with the preponderance of saturated fatty acids found in position 1 of naturally occurring glycerophospholipids. Support of this hypothesis has come from several lines of evidence. First, in Ehrlich ascites tumor cells, where the choline phosphoglycerides do not show the asymmetric distribution of fatty acids (3), the activity of the mitochondrial GAT cannot be detected (4). Secondly, in cells grown in primary culture, the mitochondrial acyltransferase activity decreases with a concomitant increase in the amount of 18:1 fatty acid in position 1 in choline phosphoglycerides (5). Finally, in the presence of bovine serum albumin (BSA), mitochondrially synthesized LPA can exit the organelles, be translocated to the microsomes and converted to phosphatidic acid by the microsomes (6). In this communication we present data that mitochondrially synthesized phospholipids also exit the organelles if BSA is replaced by the cytosolic fraction. We report here purification of a 15 kDa cytosolic protein which binds LPA, increases export of LPA from mitochondria and stimulates GAT activity.

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Abbreviations:
BSA, bovine serum albumin; GAT, glycerophosphate acyltransferase; LPA, lysophosphatidic acid; SDS-PAGE, sodium-dodecylsulfate polyacrylamide gel electrophoresis.

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Materials and Methods

Materials: Male Sprague-Dawley rats were purchased from Taconic Farms, Germantown, NY. sn-[2-^{3}H] Glycerol 3-phosphate was synthesized enzymatically from sn-[2-^{3}H] glycerol (Du Pont-New England Nuclear) and purified as described previously (4). The specific radioactivity of sn-[2-^{3}H] glycerol 3-phosphate employed was 1.13×10^4 cpm/nmol. Sephadex G-100, Sepharose Q Fast Flow and low molecular weight gel filtration calibration kit (ribonuclease A, chymotrypsinogen A, ovalbumin, albumin, Blue Dextran 2000) were obtained from Pharmacia; Biogel P-10 and silver stain kit were obtained from Biorad; cytochrome c, insulin and Lipidex (hydroxyalkoxypropyl-dextran) were purchased from Sigma and silica gel G plates without fluorescence indicator were products of Whatman. The sources of all other materials were as described previously (7).

Preparation of subcellular fractions: Liver mitochondria were prepared from 175 to 200 g male rats as previously described (1). To obtain the liver cytosolic fraction, the post-mitochondrial supernatant was centrifuged at 105,000 x g for 90 min and the supernatant liquid excluding the floating layer of fat was collected and desalted on Pharmacia PD-10 columns. Delipidation of this material was performed by passing through Lipidex column at 37° C as described (8).The cytosolic fraction, thus obtained, was either used immediately or stored in small aliquots at -70°C.

Analytical Methods: GAT activity was measured by following the incorporation of sn-[2-^{3}H] glycerol 3-phosphate into butanol-extractable phospholipids (4); however, the incubation medium did not contain asolectin or BSA. The concentration of the mitochondrial protein in the incubation medium was between 0.2 to 0.4 mg/ml. For sedimenting mitochondria from the acyltransferase assay medium, the incubated mixture was cooled to 4°C and spun at 23,000 x g for 15 min. The sediment, after suspending it in 0.5 ml water, and the supernatant fraction were extracted with butanol (4). Protein was assayed as per Lowry et al. (9) using bovine serum albumin as a standard. Sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (10) and gels were stained with silver. All other methods were as described previously (7).

Purification of LPA-binding proteins: After incubation in the acyltransferase assay medium in the presence of cytosolic fraction, the mitochondria were sedimented and the bulk amount of [2-^{3}H]-glycerophosphate was removed from the supernatant by using Pharmacia PD-10 columns previously equilibrated with 20 mM Tris/HCl buffer pH 8.4. Using Centricon 10 (Amicon) membrane filters, the sample (16 ml) was concentrated to 5 ml (total amount of protein was 32 mg) and loaded on a Sephadex G-100 column (2.5 x 55 cm) previously equilibrated with 20 mM Tris/HCl buffer, pH 8.4. The column was washed with the same buffer at 13 ml/h and 5.2 ml fractions were collected. For determination of molecular weight of peaks (ii) and (iii), the column was calibrated with Blue dextran 2000, BSA, ovalbumin, chymotrypsinogen A, ribonuclease A, cytochrome c and insulin. Separation on Sephadex G-100 column was repeated 6 times in the same manner and fractions 25-30 (peak i) and 41-46 (peak ii) were combined.

Combined fractions 25-30 from 6 runs on Sephadex G-100 column were loaded on Sepharose Q column (1.5x30 cm), equilibrated with 20 mM Tris/HCl buffer, pH 8.4. The column was washed with 50 ml of the same buffer and then eluted with 400 ml of linear gradient of 0-1 M NaCl in the same buffer. Fractions containing LPA were analyzed by SDS-PAGE.

Fractions 41-46 (peak ii) from 6 runs on Sephadex G-100 were combined, transferred to 20 mM Tris/HCl buffer, pH 9.0 and loaded on Sepharose Q column (1.5x30 cm), equilibrated with the same buffer. The column was washed with 50 ml of this buffer and then eluted with 400 ml of linear gradient of 0-0.5 M NaCl in 20 mM Tris/HCl buffer, pH 9.0. Fractions containing LPA were combined, concentrated to 2 ml using Centricon 10 membrane filters and loaded on Biogel P-10 column (1.5x95 cm). The column was eluted at 5 ml/h , 1.3 ml fractions were collected and those containing LPA were analyzed by SDS-PAGE.

In some experiments, LPA was removed from purified preparations by passing through Lipidex column (4x1 cm) at 37° C as described (8). More than 90 % of LPA was removed by this procedure and recovery of protein was more than 80%.

Results and Discussion

When mitochondria were incubated in the GAT assay medium in the presence of the 105,000 x g supernatant fraction, acylation of sn-glycerol 3-phosphate proceeded approximately up to five times faster than in its absence (Fig. 1). After sedimentation of the mitochondria from the assay mixture, the supernatant fluid and pellet were analyzed for labeled acylated product. With increasing concentration of the cytosolic proteins

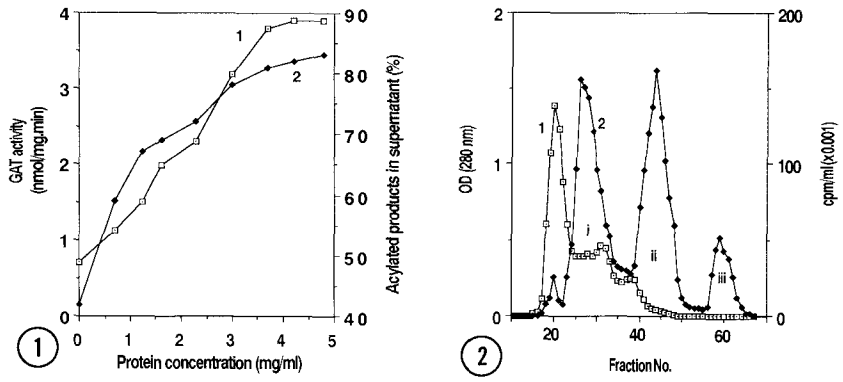

Fig. 1. GAT activity (curve 1) and phospholipid efflux from mitochondria (curve 2) as a function of concentration of 105,000 x g supernatant fraction of liver. GAT activity was determined as given in Materials and Methods section and the reaction was stopped by addition of 1-butanol. For measurement of efflux of acylated products the reaction was stopped by cooling on ice, the mitochondria were pelleted by centrifugation and phospholipids were extracted from the sediment and the supernatant fluid with 1-butanol as described [1].

Fig. 2. Gel filtration profile (Sephadex G-100) of 105,000 x g supernatant fraction of liver cytosol labeled with mitochondrially synthesized LPA. Elution of protein was monitored as absorbance at 280 nm (curve 1) and LPA was determined by scintilation counting of butanol extracts of individual fractions (curve 2).

(105,000 x g supernatant fraction) increased amounts of acylated product was found in the supernatant fluid (Fig.1).

This finding is in accordance with the previous observation that BSA extracts LPA from mitochondria and thus stimulates the synthesis of this phospholipid (6). BSA is known to bind a number of ligands like fatty acids and fatty acyl-CoAs (11). Data presented in Fig. 1 indicate that the liver cytosol may contain protein(s) acting similarly as BSA, binding LPA and stimulating GAT activity.

To determine if cytosolic proteins in general, function to extract the mitochondrially formed LPA or if there are specific proteins to do this job, mitochondria were incubated in the GAT assay medium in the presence of delipidated cytosolic fraction (105,000 x g supernatant fraction). After sedimenting the mitochondria, binding of LPA to proteins was monitored by gel filtration on calibrated Sephadex G-100 column. Fig. 2 documents the results. Labeled LPA eluted from this column in three peaks, (i), (ii) and (iii), corresponding to M_rs of 60-70 kDa, 10-20 kDa and <5 kDa, respectively. LPA eluted in peaks (i) and (ii) was associated with proteins as judged by the OD_{280} curve in Fig. 2. However, the existence of a protein or polypeptide in peak (iii) could not be determined since both low molecular weight compounds and free LPA eluted in this volume even in the absence of the cytosolic protein in the mitochondrial incubation.

The LPA-binding proteins from peaks (i) and (ii) were further purified on Sepharose Q, and Sepharose Q and Biogel P-10, respectively. Proteins from peak (i) were resolved in several protein peaks on Sepharose Q column and LPA was eluted with one of them at 0.3 M NaCl. Electrophoresis of the peak fraction with highest concentration of LPA revealed one band at ~ 70 kDa. Proteins from peak (ii) were similarly resolved on Sepharose Q column and LPA containing material was further purified on Biogel P-10. Electrophoretic analysis of the peak LPA-containing fraction revealed one band at ~ 15 kDa (Fig. 3).

When LPA was removed from the 70 kDa and 15 kDa proteins by passing them through Lipidex column at 37° C and the proteins were concentrated and incubated with mitochondria in GAT assay medium, both proteins stimulated GAT activity and export of acylated products from mitochondria. Data given in Table 1 were obtained

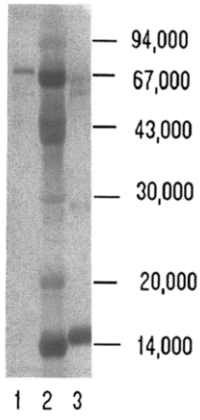

Fig. 3. Purity of the 70 kDa and 15 kDa LPA-binding proteins in SDS-PAGE, stained with silver. Lanes: 1, the 70 kDa LPA-binding protein after Sepharose Q chromatography (2 μg); 2, calibration proteins (with M_r): phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), α-lactalbumin (14,000), (15 μg); 3, the 15 kDa LPA-binding protein after Biogel P-10 chromatography (3 μg).

using saturating concentrations of both proteins, i. e. further increase in concentration of either protein did not result in additional increase of GAT activity or export of acylated products. Analysis by thin layer chromatography showed that LPA constituted more than 85 % of the acylated products released from mitochondria under these conditions.

Our results indicate that specific proteins are involved in binding and removing LPA synthesized by mitochondrial outer membrane. At present, we can only speculate what proteins are responsible for this binding. The 70 kDa protein is probably rat serum albumin (M_r=~65 kDa) which is present in liver preparations due to contamination of the liver with blood (12, 13, 14). Thus, this protein appears to stimulate mitochondrial GAT as is done by BSA (6).

The 15 kDa protein may represent a unique LPA-binding protein. However, there are also two other possible candidates which can account for the LPA-binding activity of this protein. These proteins are nonspecific lipid transfer protein and fatty acid-binding protein . Both of these proteins exhibit M_r in the range of 13-15 kDa and a broad range of substrate specificity. The nonspecific lipid transfer protein transports all natural diacylphospholipids and cholesterol (15) and has a high affinity for mitochondria (16). Liver fatty acid binding protein binds long chain fatty acids, their CoA thioesters, L-carnitine esters (15, 17), and prostaglandin

TABLE 1

Stimulation of GAT activity and export of LPA from mitochondria in the presence of LPA-binding proteins

Protein	Concentration (mg/ml)	GAT activity (nmol/mg.min)	LPA in supernatant (%)
-	-	0.8	42
70 kDa	1.2	2.1	82
15 kDa	1.1	2.6	86

GAT activity and export of LPA from mitochondria were measured as given in Fig. 1.

E_1 (18). This protein stimulates several microsomal enzymes involved in glycerolipid biosynthesis (19, 20, 21, 22). Recently, it has been found that rat liver heme-binding protein, belonging to the group of fatty acid binding proteins, stimulates the efflux of heme from mitochondria (23), which is an analogous situation to LPA export from mitochondria due to LPA-binding protein.

Although a large portion of LPA eluted in peak (iii) from Sephadex G-100 column (Fig. 2) represents probably free LPA, we cannot rule out the possibility that some low molecular weight compound might be involved in binding of this phospholipid. Involvement of low molecular weight peptides in lipid binding is known (14). However, even if there is such a peptide which binds LPA, it represents only a minor LPA binding activity in rat liver cytosol (Fig. 2).

The 15 kDa protein stimulates mitochondrial GAT by binding and removing mitochondrially synthesized LPA presumably by relieving the end-product inhibition. This protein may carry mitochondrially synthesized LPA to the endoplasmic reticulum for the synthesis of phosphatidic acid (6) and thus regulate phospholipid biosynthesis and asymmetric distribution of fatty acids in cellular glycerolipids.

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