Molecular and Cellular Biochemistry **100**: 1–8, 1991.

Original Article

Fatty acid binding protein removes fatty acids but not phospholipids from microsomes liposomes and sonicated vesicles

Rosana Zanetti* and Angel Catalá**
Cátedra de Bioquímica, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CC 296, (1900) La Plata, Argentina

Received 11 August 1989; accepted 10 April 1990

Key words: fatty acid binding protein, microsomes, liposomes, sonicated vesicles

Abstract

Evidence is provided in this paper that indicates that fatty acids but not phospholipids are removed from microsomes or artificial membranes (liposomes, unilamellar vesicles) by mouse liver cytosolic preparations enriched with fatty acid binding protein (FABP). The cytosolic proteins can act as acceptors for fatty acids but not for phospholipids of microsomal origin. Direct evidence came when liposomes made of egg yolk phosphatidylcholine, containing both [^{14}C]labeled phospholipids and [1-^{14}C] palmitic acid were incubated with FABP. Using sonicated vesicles as fatty acid or phospholipid donors, mouse liver fatty acid binding protein was capable of binding palmitic acid but not phospholipids. These studies suggest that liver fatty acid binding protein can interact with different kinds of membranes increasing specifically the desorption of fatty acids.

Abbreviations: FABP-Fatty Acid Binding Protein, PC-Phos phatidylcholine

Introduction

The movement of lipids within membranes at the intracellular level has been proposed to be mediated by specific proteins located in the cytosol [1]. A low molecular weight group of proteins (10–15 KDa) named fatty acid binding protein (FABP) are a class of relatively abundant proteins that are generally thought to be involved in the intracellular transport of fatty acids and other organic anions [2, 3]. At present it is still unclear if phospholipids are associated to FABP. Studies of Rüstow *et al.* [4] revealed such association, but others found that FABP does not bind this ligand [5, 6]. Ockner *et al.* [7] have demonstrated that the protein does not contain bound phospholipids. However, in a recent study Burrier and Brecher suggested that liver FABP may be involved in the intracellular metabolism of lysophosphatidylcholine and fatty acids [8]. There exist, therefore, confusion as to the true nature of the protein bound lipids. In the present study we have used mouse liver microsomes, liposomes and sonicated vesicles to demonstrate that preparations enriched with FABP promote the desorption of fatty acids but not phospholipids from these membranes.

* Fellow of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina
** Member of Carrera de Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET)

Materials and methods

[1-^{14}C]palmitic acid (58 mCi/mmol) was purchased from The Radioachemical Centre, Amersham. [1-^{14}C]acetic acid, sodium salt (2.5 mCi/mmol) was a product from New England Nuclear, Boston, Mass. Sephadex G75, Sepharose 4B and DEAE Sephadex A50 were from Pharmacia Fine Chemicals Inc., Piscataway, N.J.. Butylated hydroxytoluene (BHT: 2,6-Di-t-butyl-p-cresol) was purchased from Sigma Chemical Co., St. Louis, MO, USA.

Isolation of mouse liver cytosolic proteins with fatty acid binding properties

The cytosolic fraction was prepared from a 33% homogenate of mice livers in 10 mM Tris-HCl pH 7.4, 0.25 M sucrose, 10 mM EDTA, as previously described [9]. A partially purified FABP containing fraction was prepared by filtration of mouse liver cytosol in a column of Sephadex G75 (3 × 40 cm) equilibrated with 10 mM Tris-HCl pH 7.4, 0.01% NaN_3. Protein fractions (130–190 ml) were pooled and concentrated by ultrafiltration (Amicon UM-2); this fraction is named Z-protein in this work. Z-protein was applied to a DEAE-cellulose column (1 × 4 cm) equilibrated with 30 mM Tris-HCl pH 8.5. The column was initially developed with the equilibrating buffer until no protein was eluted (fraction DEI), then a second protein fraction (DEII) was obtained by elution with 0.3 M NaCl in the same buffer. Both fractions DEI and DEII were dialized against 10 mM Tris-HCl pH 7.4, 0.01% NaN_3 and concentrated by ultrafiltration.

Preparations of microsomes containing various radioactive lipids or palmitic acid

A mouse was injected (i.p.) with [1-^{14}C]sodium acetate (50 μCi). Ninety minutes after injection the liver was quickly removed, cut into small pieces and washed extensively with 0.15 M NaCl. An homogenate 30% (w/v) was prepared in soln. 0.25 M sucrose, 10 mM Tris-HCl pH 7.4, 10 mM EDTA, using a Potter-Elvejhem homogeneizer. The homogenate was spun at 10,000 g for 10 min. The resulting supernatant (4 ml) was applied to a Sepharose 4B column (1.6 × 12 cm) equilibrated and eluted with 10 mM Tris-HCl pH 7.4, 0.01% NaN_3. The microsomal fraction appearing in the void volume (10–16 ml) was brought to 0.25 M sucrose by addition of solid sucrose. The quality of this microsomal preparation is of similar composition as regards concentrations and activities of certain microsomal enzymes to that obtained by ultracentrifugation [10].

In order to obtain microsomes labeled with palmitic acid the i.p. injection was omitted and 3 ml of postmitochondrial supernatant were incubated with 2 ml of palmitic acid (sodium salt) containing 0.5 μCi of labeled fatty acid. After incubation for 10 min at room temperature the microsomes were obtained as described before. All operations were carried out at 4° C.

Removal of phospholipids and fatty acids from labeled microsomes by proteins with fatty acid binding properties

Lipid labeled microsomes (2.7 mg of protein) were incubated with soluble proteins (1–3 mg) in a total volume of 2.0 ml, 0.25 M sucrose, 10 mM EDTA, 10 mM Tris-HCl pH 7.4. Incubation was carried out for 10 min at 37° C. At the end of the incubation the pH of the medium was quickly adjusted to 5.1 by addition of 0.5 ml of 0.2 M sodium acetate-acetic acid pH 5.0. Microsomes were quantitatively sedimented at 10,000 g for 10 min. The microsomal pellets and supernatants obtained in the transfer assay system described above were extracted by the procedure of Folch *et al.* [11]. Individual lipids were separated by one-dimensional thin-layer chromatography on silica gel G60 (Merck) 0.25 mm, using petroleum ether, ethyl ether, acetic acid (90 : 10 : 1, v/v/v). The spots were visualized by iodine vapors. Selected areas were scraped from the plate and radioactivity measured by liquid scintillation counting.

Preparation of liposomes and sonicated vesicles

Appropriated volumes of the stock lipid solutions were mixed and dried at 37° C under a stream of nitrogen. The final mixture contained 5 μmol of egg yolk PC, 1 μmol of radioactive phospholipids and 0.5 μmol of [1-^{14}C]palmitic acid. The lipids were redissolved in a few ml of diethylether and dried again under nitrogen. After the addition of 1.5 ml of buffer L (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 3 mM NaN_3, pH 7.4) the suspension was shaken during 5 min on a vortex mixer.
Unilamellar vesicles were prepared by sonication of liposomes containing egg yolk PC and either radioactive phospholipids or [1-^{14}C]palmitic acid. Sonication of the liposome suspension was performed (6 min at point 3) with a probe-type Sonicator Heat-System, Inc. Model W-225R. The sonicated preparations were centrifuged at 12,000 g 10 min and used immediately.

Interaction of liposomes with Z-protein

Aliquots of liposomes (530 nmol [^{14}C]phospholipids, 42 nmol [1-^{14}C]palmitic acid) were incubated with various amounts of Z-protein, final volume 2.0 ml. After 10 min at 37° C with agitation the mixture was centrifuged at 10,000 g 10 min. The precipitate (liposomes) were resuspended in 1 ml of buffer L. The liposomes and supernatants were extracted by Folch *et al.* procedure [11]. The lipid extracts were subjected to TLC separation on 0.25 mm thick silica gel G60 plates. Plates were developed with diethylether, hexane, acetic acid (1 : 9 : 0.1, v/v/v). The spots were visualized with iodine vapors, scraped off the plates into counting vials, then 5 ml of liquid scintillation mixture (toluene 666 ml, triton X100 333 ml, omnifluor 4 g) were added and the radioactivity was counted using a liquid scintillator spectrometer.

Interaction of sonicated vesicles with Z-protein

Sonicated vesicles consisting of egg yolk PC-radioactive phospholipids or egg yolk PC-[1-^{14}C]palmitic acid (50, 25 nmol) were incubated with microsomes (1.2 mg of protein) and various amounts of Z-protein, final volume 1 ml 0.01 M Tris-HCl pH 7.4. After 10 min at 37° C the mixture was brought to pH 5.0, centrifuged at 10,000 g 10 min and the radioactivity in the supernatant determined.

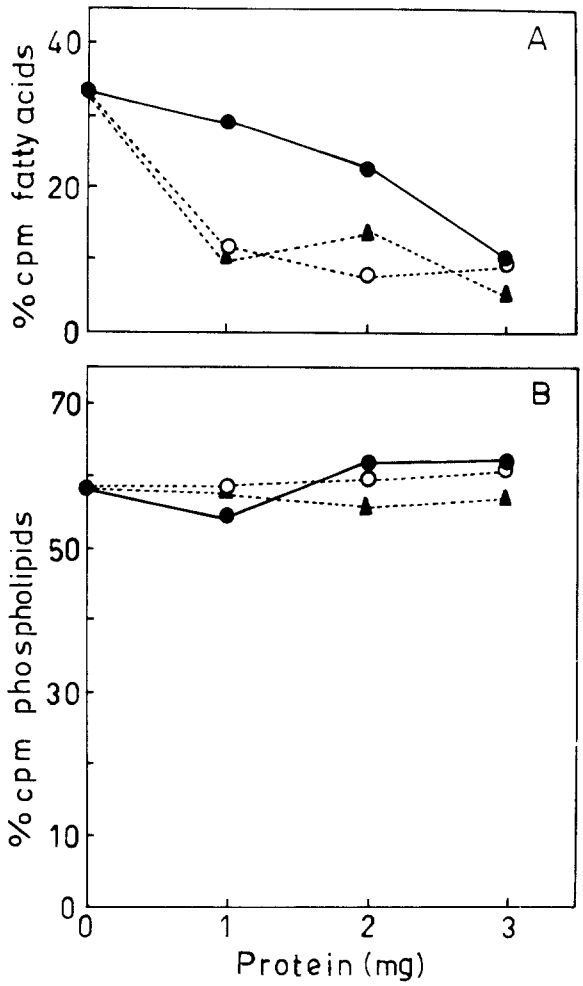

Fig. 1. Removal of phospholipids and fatty acids from mouse liver microsomes labeled in vivo. Microsomes (2.7 mg of protein) containing radioactive lipids were incubated with Z-protein (●——●), DEI (○---○) and DEII (▲---▲) in a final volume of 2.0 ml, 10 mM Tris HCl pH 7.4. After 10 min at 37° C the mixture was brought to pH 5.0 and the microsomes separated by centrifugation. The lipids were extracted and separated by TLC and the percent of radioactivity associated to fatty acids (A) and phospholipids (B) was determined as described under Materials and methods.

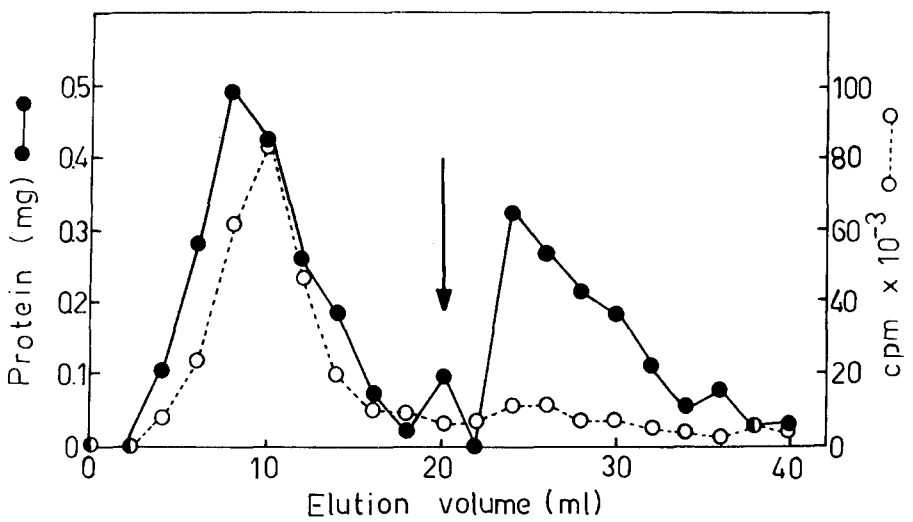

Fig. 2. Chromatography on DEAE-cellulose of Z-protein-palmitic acid complex. Column size: 1 × 4 cm; sample applied: 4.5 mg; buffer 30 mM Tris HCl pH 8.5. The arrow shows the position in which the elution was started in the presence of 0.3 M NaCl.

Other procedures

Egg yolk PC and radioactive phospholipids were prepared as described by Rusiñol *et al.* [12]. Purified PC showed a single spot on thin-layer chromatogram. The percent distribution of radioactive phospholipids analyzed by TLC was as follows: PC 47%, PE 24.3%, PI 28.7%. Both phospholipid preparations containing 0.1% butylated hydroxytoluene were stored at − 20° C. Phospholipids and proteins were determined by the methods of Chen *et al.* [13] and Lowry *et al.* [14], respectively.

Results

In this work we have investigated the specificity of the binding of phospholipids and fatty acids by fatty acid binding protein. In a preliminary experiment we tested the ability of cytosolic preparations enriched with FABP to remove phospholipids or fatty acids from mouse liver microsomes containing both radioactive species. In Fig. 1 the results of such experiment are depicted. Z-protein, DEI and DEII facilitated the removal of fatty acids from microsomal membranes. However, there was no change in the percentage of radioactive phospholipids associated to microsomes. These results indicate that the cytosolic proteins used in this study can act as acceptors for fatty acids but not for phospholipids of microsomal origin. It is important to note that either DEI or DEII remove similar amounts of fatty acids from labeled microsomes (Fig. 1A).

However, palmitic acid binding activity of these protein fractions was different after separation of Z-protein [1-^{14}C] palmitic acid complexes by ion exchange chromatography. As shown in Fig. 2, most of the protein (60%) was adsorbed weakly to the DEAE-cellulose and eluted with the equilibrating buffer (30 mM Tris-HCl pH 8.5), the remainder (40%) was eluted with the same buffer containing 0.3 M NaCl. The specific activity of DEI (143,937 cpm/mg protein) was three times higher than that of DEII (48,031 cpm/mg protein). In the next experiment, the capacity of these protein fractions for the removal of palmitic acid incorporated in mouse liver microsomes, was investigated.

Figure 3 shows that DEI was more effective than DEII for the removal of palmitic acid from microsomal membranes. Thus, when one mg of protein (DEI) was used, the rate of palmitic acid removal, was about 2.4 fold greater than using the same amount of DEII.

We also incorporated radioactive phospholipids and palmitic acid into liposomes and sonicated ves-

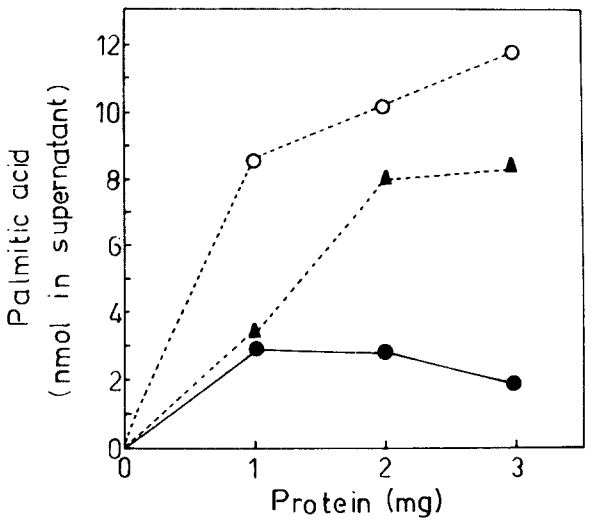

Fig. 3. Removal of palmitic acid from mouse liver microsomes labeled in vitro. Microsomes (2.5 mg of protein, 60 nmol [1-^{14}C]palmitic acid) were incubated with Z-protein (●——●), DEI (○---○) and DEII (▲---▲) in a final volume of 2.0 ml, 10 mM Tris HCl pH 7.4. After 10 min at 37° C the mixture was brought to pH 5.0 and the microsomes and supernatant was separated at 10,000 × g 10 min. The radiolabeled ligand bound to protein was determined as described under Materials and methods.

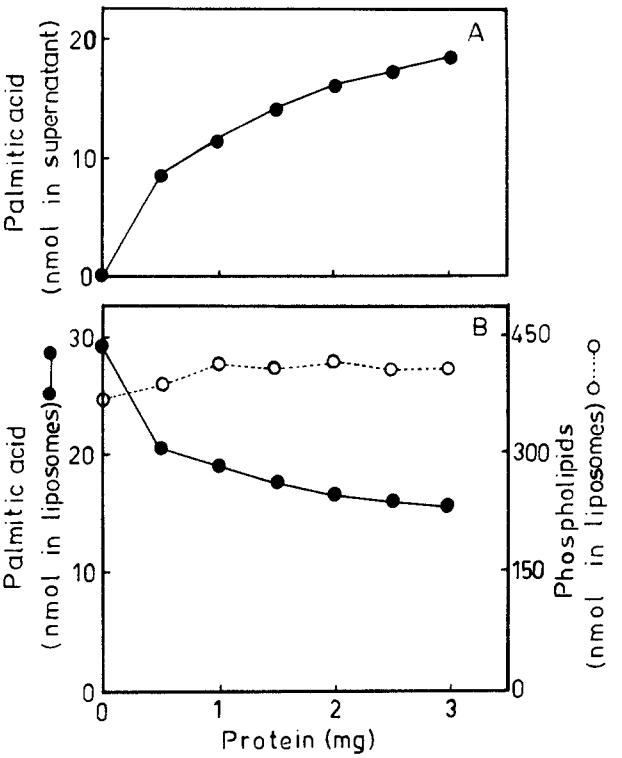

Fig. 4. Interaction of liposomes containing radioactive phospholipids and [1-^{14}C]palmitic acid with Z-protein. Liposomes consisting of egg yolk phosphatidylcholine, radioactive phospholipids and [1-^{14}C]palmitic acid (molar ratio: 1 : 0.2 : 0.1) were incubated with various amounts of Z-protein for 10 min at 37° C. After centrifugation at 10,000 × g 10 min, radioactivity present in phospholipids and fatty acids was measured as described in the text. A: supernatant; B: liposomes.

icles to determine whether Z-protein could facilitate the removal of this compounds. Z-protein stimulates palmitic acid removal from multilamellar vesicles, since there is a considerable decrease of palmitic acid associated to liposomes (Fig. 4B) that appear bound to soluble protein in supernatant (Fig. 4A). However, the removal of radioactive phospholipids incorporated together with fatty acid in the same vesicles was not observed.

In the next series of experiments we investigated whether Z-protein could be important in the specific removal of [1-^{14}C]palmitic acid or radioactive phospholipids incorporated separately in small unilamellar vesicles. For this purpose, sonicated vesicles made of egg yolk PC, containing equal amounts of radioactive phospholipids or palmitic acid, were incubated with mouse liver microsomes and Z-protein. After separation by centrifugation it was observed that more than 97% of the radioactivity was in the pellet, which indicated that the vesicles were co-precipitated with the microsomes. Figure 5 shows the percentage of radioactivity in the supernatant after interaction of microsomes with both types of sonicated vesicles and Z-protein. In the absence of protein, 1.27 and 2.88% of the total radioactivity remains in the supernatant when vesicles containing radioactive phospholipids or palmitic acid were used. This is an indication that vesicles possibly interact by either adsorption or fusion with microsomes. The addition of Z-protein facilitated the removal of palmitic acid from the precipitated vesicles, whereas a small amount of radioactive phospholipids appear in the soluble. Thus, one mg of soluble protein removes 1.46 nmol of palmitic acid, whereas only 0.17 nmol of phospholipids were removed by the same amount of protein.

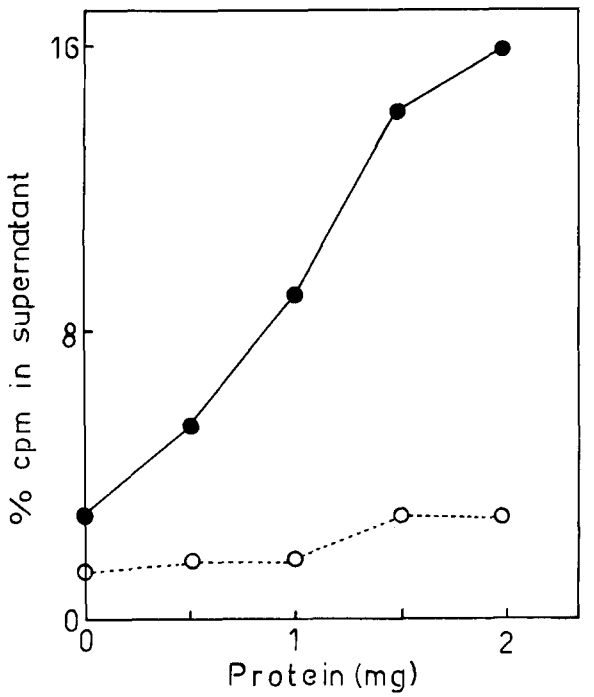

Fig. 5. Interaction of sonicated vesicles containing radioactive phospholipids or [1-^{14}C]palmitic acid with Z-protein. Sonicated vesicles consisting of egg yolk phosphatidylcholine-radioactive phospholipids or egg yolk phosphatidylcholine-[1-^{14}C]palmitic acid (50,25 nmol) were incubated with microsomes (1.2 mg of protein) and several amounts of Z-protein. After 10 min at 37° C the mixture was brought to pH 5.0, centrifuged at 10,000 × g 10 min and the radioactivity in the supernatant determined. Radioactive phospholipids (O---O); [1-^{14}C]palmitic acid (●—●).

Discussion

Fatty acid binding protein modulates the activity of a number of enzymes involved in fatty acid metabolism [15, 16], for this reason it is important to know what kind of lipids are bound to its molecule.

By using different approaches we have found that Z-protein, DEI and DEII removed specifically labeled fatty acids from microsomal membranes or palmitic acid from microsomes and sonicated vesicles, whereas phospholipid removal was not observed. As described in Introduction, the presence of phospholipids associated to fatty acid binding protein is still a point of controversy. Although these contradictory findings may be due to the use of different types of analysis, it should also be realized that a different methodology was applied in our studies.

In initial experiments using mouse liver microsomes labeled in vivo we have shown that fatty acids but not phospholipids were removed by a FABP containing preparation.

Previous studies have shown liposomes to be a convenient donor of fatty acids in an aqueous assay, since they provided a means of adding relatively high concentrations of fatty acid which was accesible to binding by either albumin or liver FABP [17].

In order to prove more unequivocally whether or not phospholipids could be associated to FABP, liposomes containing both radioactive phospholipids and [1-^{14}C]palmitic acid were prepared. The use of liposomes resulted in rates of palmitic acid removal by Z-protein that were similar to those obtained when vesicles were used, 9 and 11.7 nmol respectively. However, there was a little difference on the removal of phospholipids when liposomes or vesicles were used. In the presence of sonicated vesicles, FABP promotes the desorption of 0.17 nmol of phospholipids per mg of protein, whereas no desorption was observed when liposomes were used. A possible explanation for the differences observed may be due to the greater amount of phospholipids located on the outermost leaflets of vesicles than on the outermost leaflets of liposomes. In sonicated vesicles the outermost leaflets comprise 67% of their total phospholipids [18], while in liposomes about 10% of the total phospholipids are on the outer leaflet [19]. This is consistent with our observation that a crude preparation of FABP (Z-protein) or protein fractions (DEI or DEII) prepared by ion exchange chromatography were able to remove fatty acids from labeled microsomes whereas phospholipids removal was not observed.

The absence of phospholipid binding to liver fatty acid binding protein indicates that functional differences may accompany the observed dissimilarities already described between this protein and phospholipid carrier protein [20].

On the other hand, it has been demonstrated that the behaviour of Z-protein on ion exchange chromatography is unusual with respect to the pH

required to retain this protein on the column. Studies of Trulzsch and Arias [21] and those of Takahashi *et al.* [6] have demonstrated that on DEAE-cellulose chromatography at pH 8.5, Z-protein can be resolved in several peaks that are bound to the anion exchange cellulose. However, in a recent study Burrier and Brecher [8] resolved Z-protein into two main protein fractions using DE-52 chromatography. Both fractions as indicated by those authors, contain proteins with fatty acid binding properties. Our results clearly show that either DEI, eluted from the column in the absence of salt or DEII eluted in the presence of 0.3 M NaCl contain proteins with fatty acid binding properties as it was demonstrated during the interaction of those fractions with mouse liver microsomes containing palmitic acid or different lipidic species (Fig. 3 and Fig. 1). The differences observed upon the removal of fatty acids from microsomal membranes labeled *in vivo* or *in vitro* may be due to the fact that microsomes labeled *in vivo* contain many radioactive fatty acids, while microsomes labeled *in vitro* contain only radioactive palmitic acid.

The selective removal of fatty acids by preparations enriched in FABP, using both liver microsomes and phospholipid vesicles are specific with regard to FABP but not affected by the kind of membrane preparation used.

Ockner *et al.* [7] demonstrated that in rat liver, 60% of the cytosolic long-chain fatty acids, is associated with the FABP-containing fraction. Fifty to seventy percent of these endogenous fatty acids are unsaturated: oleate, linoleate and arachidonate. Considering that FABP plays a central role in cellular lipid metabolism [15], further studies are required to determine precisely what kind of fatty acids are removed from microsomal membranes by this cytosolic protein.

Acknowledgements

This study was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina.

References

1. Zilversmit DB: Lipid transfer proteins. J Lipid Res 25: 1563–1569, 1984
2. Ockner RK, Manning JA, Poppenhausen RB, Ho WKL: A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium, and other tissues. Science, Wash. 177: 56–58, 1972
3. Mishkin S, Stein L, Gatmaitan Z, Arias IM: The binding of fatty acids to cytoplasmic proteins: binding to Z protein in liver and other tissues of the rat. Biochem Biophys Res Commun 47: 997–1003, 1972
4. Rüstow B, Risse S, Kunze D: Endogenes lipidmaster, organ verteilung und diatbeeinflussung einer fettsaurebindenden proteinfraktion des leberzytosols der ratte. Acta Biol Med Ger 41: 439–445, 1982
5. Burnett DA, Lysenko N, Manning JA, Ockner RK: Utilization of long chain fatty acids by rat liver: studies of the role of fatty acid-binding protein. Gastroenterology 77: 241–249, 1979
6. Takahashi K, Odani S, Ono T: Isolation and characterization of the three fractions (DE-I, DE-II and DE-III) of rat liver Z-protein and the complete primary structure of DE-II Eur J Biochem 136: 589–601, 1983
7. Ockner RK, Manning JA, Kane JP: Fatty acid binding protein. Isolation from rat liver, characterization and immuno-chemical quantification. J Biol Chem 257: 7872–7878, 1982
8. Burrier RE, Brecher P: Binding of lysophosphatidylcholine to the rat liver fatty acid binding protein. Biochim Biophys Acta 879: 229–239, 1986
9. Zanetti R, Catalá A: Lipid binding properties of fatty acid binding protein isolated from mouse liver. Acta Physiol Pharmacol Latinoam 38: 135–144, 1988
10. Tangen O, Jonsson J, Orrenius S: Isolation of rat liver microsomes by gel filtration. Analyt Biochem 54: 597–603, 1973
11. Folch J, Lees M, Sloane Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497–509, 1957
12. Rusiñol A, Salomón RA, Bloj B: Phospholipid transfer activities in toad oocytes and developing embrios. J Lipid Res 28: 100–107, 1987
13. Chen PS Jr, Toribana TY, Warner H: Microdetermination of phosphorus. Analyt Chem 28: 1756–1758, 1956
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275, 1951
15. Swetser DA, Heuckeroth RO, Gordon JI: The metabolic significance of mammalian fatty-acid-binding proteins: Abundant proteins in search of a function. Ann Rev Nutr 7: 337–359, 1987
16. Catalá A: Stearic acid desaturation in rat liver microsomes: stimulation by fatty acid binding protein. Acta Physiol Pharmacol Latinoam 36: 19–27, 1986
17. Brecher P, Saouaf R, Sugarman JM, Eisenberg D, LaRosa

K: Fatty acid transfer between multilamellar liposomes and fatty acid binding proteins. J Biol Chem 259: 13395–13401, 1984

18. Mason JT, Huang C: Hydrodynamic analysis of egg phos phatidylcholine vesicles. Ann N Y Acad Sci 308: 29–49, 1978
19. Bangham AD, De Gier J, Greville GD: Osmotic properties and water permeability of phospholipid liquid crystals. Chem Phys lipids 1: 225–246, 1967
20. Glatz JFC, Veerkamps JM: Intracellular fatty acid-binding proteins. Int J Biochem 17: 13–22, 1985
21. Trulzsch D, Arias IM: Z protein: Isolation and characterization of multiple forms in rat liver cytosol. Arch Biochem Biophys 209: 433–440, 1981

Address for offprints: A. Catalá, Cátedra de Bioquímica, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CC 296, (1900) La Plata, Argentina