Ligand-dependent interaction of hepatic fatty acid-binding protein with the nucleus

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Abstract Our studies were conducted to explore the role of hepatic fatty acid-binding protein (L-FABP) in fatty acid transport to the nucleus. Purified rat L-FABP facilitated the specific interaction of [^{3}H]oleic acid with the nuclei. L-FABP complexed with unlabeled oleic acid decreased the nuclear association of [^{3}H]oleic acid:L-FABP; however, oleic acid-saturated bovine serum albumin (BSA) or fatty acid-free L-FABP did not. The peroxisome-proliferating agents LY171883, bezafibrate, and WY-14,643 were also effective competitors when complexed to L-FABP. Nuclease treatment did not affect the nuclear association of [^{3}H]oleic acid:L-FABP; however, proteinase treatment of the nuclei abolished the binding. Nuclei incubated with fluorescein-conjugated L-FABP in the presence of oleic acid were highly fluorescent whereas no fluorescence was observed in reactions lacking oleic acid, suggesting that L-FABP itself was binding to the nuclei. The nuclear binding of FABP was concentration dependent, saturable, and competitive. LY189585, a ligand for L-FABP, also facilitated the nuclear binding of fluorescein-conjugated L-FABP, although it was less potent than oleic acid. A structural analog that does not bind L-FABP, LY163443, was relatively inactive in stimulating the nuclear binding. Potential interactions between L-FABP and nuclear proteins were analyzed by Far-Western blotting and identified a 33-kDa protein in the 500 mM NaCl extract of rat hepatocyte nuclei that bound strongly to biotinylated L-FABP. Oleic acid enhanced the interaction of L-FABP with the 33-kDa protein as well as other nuclear proteins. We propose that L-FABP is involved in communicating the state of fatty acid metabolism from the cytosol to the nucleus through an interaction with lipid mediators that are involved in nuclear signal transduction.—Lawrence, J. W., D. J. Kroll, and P. I. Eacho. **Ligand-dependent interaction of hepatic fatty acid-binding protein with the nucleus. *J. Lipid Res.* 2000.** 41: **1390–1401.**

Supplementary key words fatty acid-binding protein • LY171883 • fatty acids • bezafibrate • oleic acid • WY-14,643 • peroxisome proliferators • nuclear transport

Long-chain fatty acids are poorly soluble in an aqueous environment and utilize intracellular binding proteins to increase their solubility and facilitate movement through the cytoplasm (1–4). Fatty acid-binding protein (FABP) is a 14-kDa protein that is a member of a family of proteins participating in fatty acid metabolism in numerous tissues including heart, intestine, brain, and liver (4, 5). Liver FABP (L-FABP) is found mainly in the cytosol, accounting for up to 5% of the cytoplasmic protein in hepatocytes (6). This protein is thought to protect the cell from the detergent effects of fatty acids and promote the solubility of fatty acids in the cellular aqueous environment (5). It is also reported to transport fatty acids to sites of metabolism throughout the cell, including microsomes and mitochondria (7–9). Interestingly, L-FABP binds other small hydrophobic molecules including peroxisome proliferators (5, 10–12), suggesting that L-FABP may play a role in the biological actions of these agents.

In addition to their role in lipid synthesis and energy metabolism, long-chain fatty acids regulate the expression of several genes involved in lipid metabolism including apolipoprotein A-I, low density lipoprotein receptors, glucose-6-phosphate dehydrogenase, and fatty acid synthase (13–16). The control of gene expression by fatty acids is currently thought to be mediated by ligand-dependent transcription factors including the peroxisome proliferator-activated receptor (PPAR) and the fatty acid-activated receptor (FAAR), members of the steroid hormone receptor superfamily (17–20). These transcription factors are located in the nucleus (21). Thus, transport of the fatty acids from the cytoplasm to the nucleus would seem to be required for interaction with

Abbreviations: BEZA:FABP, bezafibrate-saturated fatty acid-binding protein; bFABP, biotin-conjugated FABP; BSA, bovine serum albumin; CRBP, cellular retinol-binding protein; CTBP, cellular thyroxine-binding protein; FAAR, fatty acid activated receptor; HRP, horseradish peroxidase; FAF, fatty acid free; FLUOS-FABP, fluorescein-conjugated FABP; L-FABP, liver fatty acid-binding protein; LY:FABP, LY171883-saturated FABP; O:BSA, oleic acid-saturated BSA; O:FABP, oleic acid-saturated FABP; PPAR, peroxisome proliferator activated receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WY:FABP, WY-14,643-saturated FABP.

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nuclear transcription factors. L-FABP is an obvious candidate for this transport function. Tissue-specific FABPs have been observed in the nucleus of bovine and rat hepatocytes, bovine heart, brain granule neurons, and in locust flight muscle (22–27). L-FABP is reported to have a role in cellular growth and differentiation (28, 29). It promoted the growth of transfected hepatomas cells during exposure to fatty acids or peroxisome proliferators (30). Growth-modulating eicosanoids are potent ligands for L-FABP (10, 11, 31, 32). Thus, it is reasonable to hypothesize that L-FABP has a role in regulating gene expression by transporting bioactive ligands to the nucleus. Clarke and Jump (33) postulated that L-FABP participates in the regulation of gene expression by shuttling fatty acids to a specific nuclear binding proteins such as PPARs. Evidence of such a nuclear transport function was found in the case of cellular retinol-binding protein, CRBP (34, 35), which shares structural homology with L-FABP. Likewise, the cytoplasmic binding protein, cellular thyroxine-binding protein (CTBP), was found to function in the delivery of its ligand to the nucleus (36, 37).

We show in this report that [^{3}H]oleic acid interacts with rat liver nuclei in a specific manner when complexed to L-FABP. After labeling the protein with fluorescein, we found that L-FABP interacts directly with rat liver nuclei in a specific, ligand-dependent manner. We have detected several nuclear proteins that bind L-FABP. These data provide evidence that L-FABP facilitates the transport of fatty acids to the nucleus and may communicate the state of fatty acid metabolism from the cytosol to the nucleus.

MATERIALS AND METHODS

Materials

[^{14}C]oleic acid (50 Ci/mol) and [^{3}H]oleic acid (14 Ci/mmol) had a purity of greater than 99% and were obtained from New England Nuclear (Boston, MA). Lipidex-1000 was obtained from Packard (Meriden, CT). Biotin-*N*-hydroxysuccinimide ester (NHS-biotin) and 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (NHS-FLUOS) labeling kits were obtained from Boehringer Mannheim (Indianapolis, IN). DNase I, RNase T1, RNase A, and *Hae*III were obtained from GIBCO-BRL (Gaithersburg, MD). Bovine serum albumin (type V, fatty acid free), oleic acid (99% purity), spermine, spermidine, trypsin, trypsin inhibitor, Triton X-100, and bezafibrate were obtained from Sigma (St. Louis, MO). [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (WY-14,643) was obtained from Chemsyn Laboratories (Lenexa, KS). 1-[2-Hydroxy-3-propyl-4-[4-(1*H*-tetrazol-5-yl)butoxy]phenyl]ethanone (LY171883), 1-[2-hydroxy-3-propyl-4-[[3-(1-*H*-tetrazol-5-ylmethyl)phenoxy]methyl]phenyl]ethanone (LY189585), and 1-[2-hydroxy-3-propyl-4-[[4-(1*H*-tetrazol-5-ylmethyl) phenoxy] methyl]phenyl]ethanone (LY163443) were synthesized at Lilly Research Laboratories (Indianapolis, IN). All other chemicals were of the highest quality commercially available.

Animals

Male weanling Fisher 344 rats were obtained from Harlan-Sprague Dawley (Indianapolis, IN). All animals were individually housed in stainless steel cages with a 12-h light/dark cycle and allowed free access to food and water. Livers were excised from pentobarbital-anesthetized rats and were used immediately or frozen in liquid nitrogen and stored at −70°C.

Purification of L-FABP from rat liver

Fatty acid-binding protein was purified from rat liver as described by Wilkinson and Wilton (38), using [^{14}C]oleic acid as a tracer. Purity was determined to be greater than 95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (39). Fatty acids were removed from the protein by elution through a Lipidex-1000 column at 37°C (40). The identity of L-FABP was confirmed with a specific antibody to L-FABP [kindly provided by N. Bass, University of California at San Francisco (UCSF)]. Ligand-binding constants for the purified protein were consistent with those reported previously (12).

L-FABP complexed with [^{3}H]oleic acid and peroxisome proliferators

Approximately 2 mg of delipidated L-FABP (0.5 mg/ml) was incubated with 100 μM [^{3}H]oleic acid (100 Ci/mol specific activity) at 37°C for 60 min. Free [^{3}H]oleic acid was separated from [^{3}H]oleic acid:L-FABP complexes ([^{3}H]O:FABP) by dialysis (molecular weight cutoff, 10,000) at 4°C overnight in 10 mM $NaPO_4$, pH 7.5, containing 0.2% NaN_3 to decrease the nonspecific binding to the filter apparatus. After dialysis, the specific activity was found to be more than 150 Ci/mol protein (approximately 1.5 mol of oleate per mole of FABP). Nonradioactive oleic acid and peroxisome proliferators were complexed to L-FABP for use in competition experiments by placing delipidated L-FABP (0.5 mg/ml) inside the dialysis cassette and dialyzing against a 1,000-fold excess volume of phosphate-azide buffer containing the 10 μM oleic acid, 100 μM WY-14,643, 100 μM bezafibrate, or 500 μM LY171883 at 4°C overnight. These ligand-FABP complexes were then concentrated with a Centricon (Amicon, Beverley, MA) C10 filter unit.

Covalent modification of L-FABP

Delipidated L-FABP (1 mg) was labeled with NHS-FLUOS or NHS-biotin with the respective kits from Boehringer Mannheim as described by the manufacturer. Modification with either biotin or fluorescein did not alter the affinity of L-FABP for oleate (data not shown) as described in ref. 12.

Isolation of rat liver nuclei

Nuclei were isolated as described by Luderus et al. (41). Briefly, livers from Fischer 344 male rats were homogenized in 10 volumes of 7.5 mM Tris-HCl, pH 7.5, containing 0.1 mM spermine, 0.25 mM spermidine, 40 mM KCl, 1 mM EDTA, and 2 M sucrose. Homogenates were layered over 10 ml of homogenization buffer and centrifuged at 70,000 *g* for 30 min. Pellets were rehomogenized and centrifuged as described above. Nuclei were stored at −70°C in 7.5 mM Tris-HCl, pH 7.5, 0.1 mM spermine, 0.25 mM spermidine, 40 mM KCl, 1 mM EDTA, 2 M sucrose mixed 1:1 with 100% glycerol. Before use, nuclei were washed in binding buffer (20 mM Tris-HCl, pH 7.5, containing 20 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$, 0.05 mM spermine, and 0.125 mM spermidine) containing 1% Triton X-100 on ice for 15 min. Triton X-100 was included in the wash buffer to prevent aggregation of nuclei and to remove nuclear membrane lipids (35). The nuclei were then pelleted and resuspended in binding buffer without Triton X-100. The purity of the nuclei was analyzed by light microscopy and contamination by mitochondria was assessed by cytochrome *c* oxidase activity (42). Nuclear preparations that were found to be granular or contain significant amounts of cyanide inhibitable cytochrome *c* oxidase activity were discarded. DNA content was quantified by the H33258 fluorescent assay (43).

[^{3}H]oleate:L-FABP binding to nuclei

Triton-washed nuclei were incubated with [^{3}H]O:FABP in binding buffer containing 0.2% bovine gamma globulin (120-μl

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total volume) at room temperature for 50 min with occasional mixing. Samples were then cooled on ice for 10 min before filtering a 100-μl aliquot through a 0.65-μm pore size DVPP 96-well filter plate with a Millipore (Danvers, MA) multiscreen assay system vacuum manifold. Filters were immediately washed with 100 μl of ice-cold binding buffer and the filters were dried, punched into scintillation vials, and counted. In some experiments, nuclei were treated with 200 U of DNase I, 10 U of the restriction enzyme *Hae*III, or 10 U of RNase (1:10 A/T1 ratio) for 30 min at 37°C prior to the binding reaction. Nuclease activity was confirmed by electrophoresis of SDS-lysed samples in a 0.8% agarose gel containing 0.5× TBE at 80 V for 2 h. Gels were stained with ethidium bromide and photographed under UV illumination. In some experiments, DNase-treated nuclei were further digested with trypsin (100 μg/ml) at 37°C for 60 min before addition of trypsin inhibitor (250 μg/ml).

Fluorescence microscopy

Triton-washed nuclei were resuspended in binding buffer containing 0.2% fatty acid-free bovine serum albumin (BSA). Binding buffer containing 0.2% fatty acid-free BSA, FLUOS-FABP (140 nmol), and either ethanol or oleic acid (0.5 mM) was mixed and incubated at 37°C for 10 min to allow binding of the fatty acid to the protein (100-μl total volume). Nuclei (3.3 μg of DNA) were then added and incubated at 25°C for 30 min before examination under a Leitz Laborlux D fluorescent microscope.

FLUOS-FABP binding to nuclei

Triton-washed nuclei were resuspended in binding buffer containing 0.2% fatty acid-free BSA (as described above). FLUOS-FABP, binding buffer containing fatty acid-free BSA (0.2%), and fatty acid (when appropriate) were mixed and incubated for 10 min at 37°C to allow binding of the fatty acid to the protein. Nuclei were then added and incubated at 25°C for 30 min. A 70-μl aliquot of the sample (100-μl total volume) was then loaded into a well of a 96-well Fluoricon-GF assay plate (IDEXX Laboratories, Westbrook, ME). Free L-FABP was separated from nuclei by vacuum filtration and the fluorescence quantified on a Pandex (Mundelein, IL) Screen Machine.

Fractionation of rat hepatocytes

Rat hepatocytes were prepared as previously described (44). Hepatocytes were washed twice with phosphate-buffered saline (PBS) and resuspended in lysis buffer [30 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were lysed with 1% Triton X-100 and incubated on ice for 15 min and microcentrifuged at 3,000 rpm for 5 min at 4°C. Supernatants were saved and the resulting nuclear pellet was washed with lysis buffer. The pellet was then sequentially extracted with lysis buffer containing 150, 300, and 500 mM NaCl. Samples were stored at −20°C until analyzed.

SDS-PAGE and Far Western analysis

Proteins were resolved through 12% SDS-polyacrylamide gels as described by Laemmli (39). Samples were mixed with 0.25 volume of 4× loading buffer [250 mM Tris-HCl (pH 6.8), 40% glycerol, 8% SDS, 40 mM dithiothreitol (DTT), 0.04% bromophenol blue], boiled for 2 min, and electrophoresed at 40 mA/gel. Proteins were then transferred to nitrocellulose in Tris-glycine buffer at 150 mA overnight at 4°C (45). Far Western analysis was performed as described by Hoeffler, Lustbader, and Chen (46), using a biotin-labeled L-FABP probe. Blots were blocked in TNE-50 [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA] containing 2% BSA, 1 mM DTT, and 0.02% NaN_3 at room temperature for 2 h. Blots were rinsed in TNE-50 and incubated in binding buffer (TNE-50 containing 0.2% BSA, 0.02% NaN_3). Biotinylated-FABP was bound to the blots at 37°C at a concentration of 200 ng/ml for 2.5 h. Blots were washed three times for 15 min with TNE-50 at room temperature. The biotin was detected with an avidin-HRP conjugate and an ECL chemiluminescent kit from Amersham (Arlington Heights, IL), following the manufacturer instructions. Densitometric analysis of lumigrams was performed with an LKB (Uppsala, Sweden) Ultrascan XL scanning laser densitometer.

RESULTS

Fatty acid-binding protein facilitates the specific interaction of fatty acids with the nucleus

Initial experiments were conducted to determine if free oleic acid interacted with specific sites in the nucleus. We were unable to demonstrate specific binding of free [^{3}H]oleic acid, using a variety of techniques including filtration and centrifugation. In a filtration assay, the free oleate binds to the filter apparatus and precludes the assessment of nuclear binding. In a centrifugation assay, binding of [^{3}H]oleic acid associated with rat liver nuclei in a concentration-dependent manner, but this was determined to be nonspecific because it was not inhibited by an excess of unlabeled oleate (data not shown). In contrast, the binding that was observed when [^{3}H]oleic acid was complexed to L-FABP ([^{3}H]O:FABP) was inhibited by an excess of unlabeled oleic acid:FABP (**Fig. 1A**). The binding that was observed in the presence of 4 μM protein was inhibited more than 90% by 17 μM unlabeled O:FABP. The specific binding increased as a function of the concentration of [^{3}H]O:FABP that was incubated with rat liver nuclei (Fig. 1B). The maximum specific binding was observed at an L-FABP concentration of approximately 10 μM. At saturation, the specific binding was found to be three times the nonspecific binding and was less than 6% of total radioactivity present in the reaction. [^{3}H]O:FABP associated rapidly with nuclei, reaching a steady state within 5 min (Fig. 1C). The association of [^{3}H]O:FABP increased as a function of the quantity of nuclei (DNA) in the reaction (Fig. 1D). The maximum association was observed in the presence of 3 μg of DNA.

The specificity of the association of [^{3}H]O:FABP with nuclei was further examined in competition experiments. Whereas unlabeled oleic acid:FABP inhibited the binding of [^{3}H]O:FABP to nuclei by more than 90%, fatty acid-free L-FABP was a poor competitor, causing only a 25% decrease (**Fig. 2A**). To determine if the nuclear association of [^{3}H]oleate simply required complexing with a protein, bovine serum albumin was saturated with oleic acid (O:BSA) and tested as a competitor (Fig. 2B). The O:BSA did not compete with [^{3}H]O:FABP for nuclear binding. The further addition of unlabeled O:FABP to this reaction abolished binding. In contrast to the fatty acid-complexed BSA, fatty acid-free BSA effectively prevented the nuclear binding of [^{3}H]oleate.

Nuclear binding of [^{3}H]O:FABP is inhibited by proteinase but not by nuclease treatment

It was suggested by others (47) that a potential DNA-binding motif in FABP is involved in the nuclear localiza-

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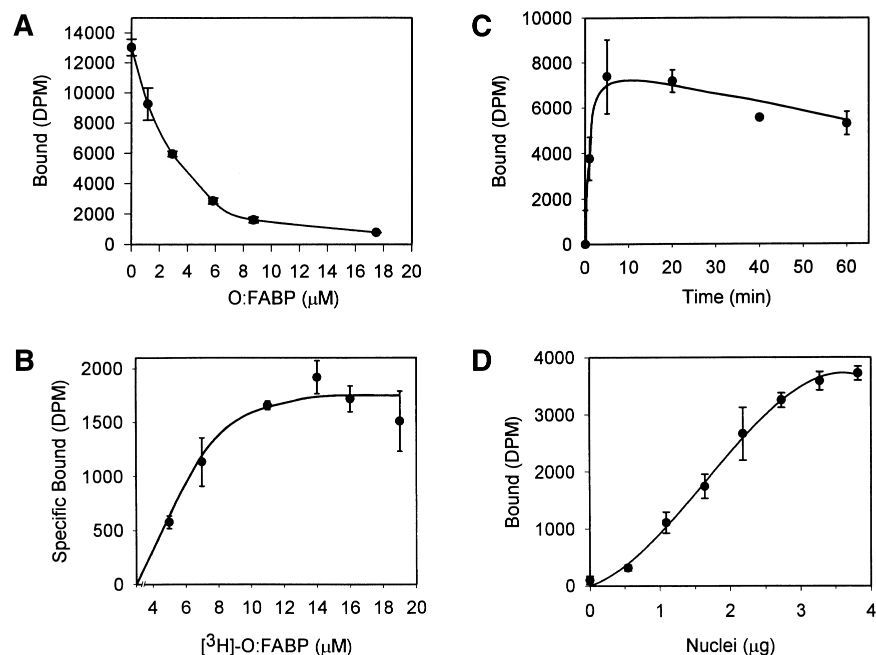

Fig. 1. Binding of [^{3}H]oleic acid:L-FABP complexes to rat liver nuclei. (A) The binding of [^{3}H]oleic acid:FABP ([^{3}H]O:FABP) to rat liver nuclei was determined by incubating nuclei (2 μ of DNA) at room temperature for 50 min with 4 μM [^{3}H]O:FABP and increasing concentrations of unlabeled oleic acid:FABP (O:FABP). Total binding was determined by filtration as described in Materials and Methods. (B) Specific binding, defined as the total binding minus the nonspecific binding measured in the presence of an excess (100 μM) of unlabeled O:FABP, was determined by incubating nuclei (2 μg of DNA) at room temperature for 50 min with increasing concentrations of [^{3}H]O:FABP. (C) The time course of the specific binding to nuclei was determined at 4°C with 4 μM [^{3}H]O:FABP. (D) The dependence of specific binding on the amount of nuclei was determined at room temperature for 50 min with 2.3 μM [^{3}H]O:FABP. Values are expressed as the average ± standard error of three determinations.

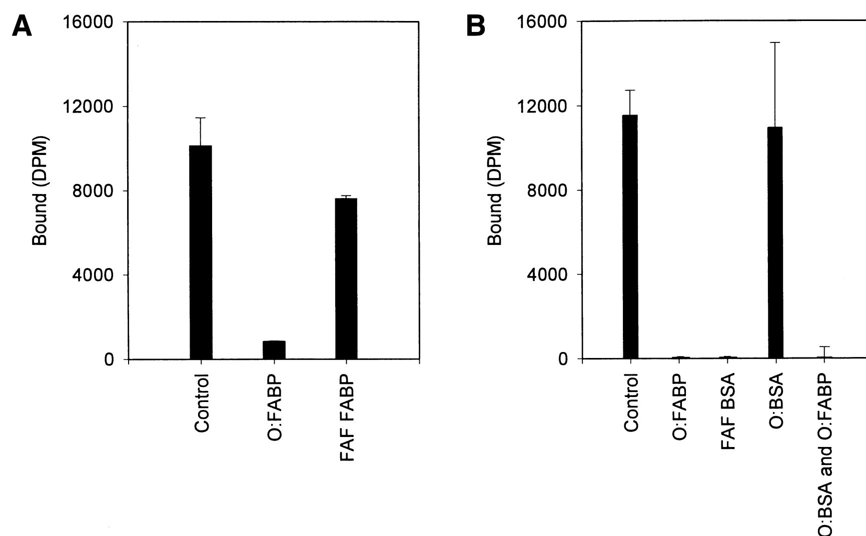

Fig. 2. Oleate-saturated BSA and fatty acid-free L-FABP do not compete with [^{3}H]oleic acid:L-FABP for nuclear binding. Rat liver nuclei (1.5 μg of DNA) were incubated with 4 μM [^{3}H]O:FABP at room temperature for 50 min. (A) In the absence or presence of 20 μM unlabeled O:FABP or 25 μM fatty acid-free L-FABP (FAF-FABP). (B) In the presence or absence of 20 μM unlabeled O:FABP, 10 μM fatty acid-free BSA, 10 μM BSA in the presence of 100 μM oleic acid (in ethanol), a combination of BSA with 100 μM oleic acid, and 20 μM unlabeled O:FABP. The binding of [^{3}H]O:FABP was determined by filtration. Values are expressed as the average ± standard error of at least three determinations.

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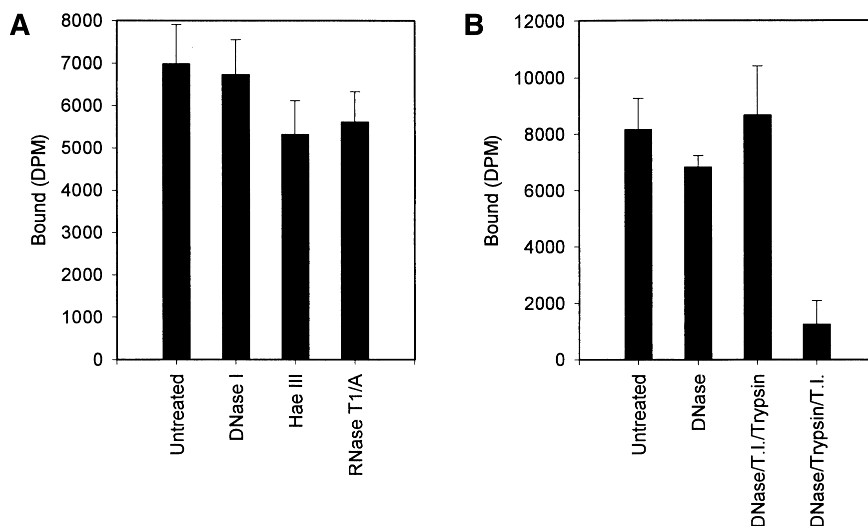

Fig. 3. Nuclear binding of [^{3}H]oleic acid:L-FABP is resistant to nuclease digestion but sensitive to proteinase digestion. (A) Nuclei (2 μg of DNA) were incubated at 37°C with either 200 units of DNase I, 10 units of *Hae*III, or 10 units of RNase for 30 min. The binding buffer containing 4 μM [^{3}H]O:FABP was then added and binding to the nuclei was determined by filtration. (B) Nuclei (2 μg of DNA) were incubated at 37°C with 200 units of DNase I for 30 min, after which the nuclei were incubated with trypsin (100 μg/ml) at 37°C for 60 min. Prior to the addition of [^{3}H]O:FABP, trypsin inhibitor (250 μg/ml) was added to prevent digestion of L-FABP. As a control, some nuclei were treated with trypsin inhibitor prior to digestion with trypsin. Binding was determined at room temperature for 50 min with 4 μM [^{3}H]O:FABP. Values are expressed as the average ± standard error of three determinations.

tion of cardiac FABP. To evaluate whether DNA was involved in the nuclear association of [^{3}H]O:FABP, nuclei were treated with *Hae*III to cut the DNA into smaller fragments, or with DNase I for maximum digestion. Nuclei were also treated with RNase as a control. Nuclease treatment altered the morphology of the nuclei but the nuclear shells retained binding activity equivalent to that of the untreated nuclei (**Fig. 3A**). When the DNase treatment was followed by trypsin (100 μg/ml), a marked decrease in binding occurred (Fig. 3B). In the latter experiment, trypsin inhibitor (250 μg/ml) was added after the proteinase pretreatment to prevent degradation of the [^{3}H]O:FABP complex. When the trypsin inhibitor was added before the proteinase preincubation, no decrease in binding occurred. Thus, degradation of nuclear protein prevented the interaction with [^{3}H]O:FABP. We were unable to test the effects of proteinase treatment alone because the released chromatin interfered with the proper filtration of the samples.

Fatty acid-binding protein binds directly to nuclei in a specific, ligand-dependent manner

To directly evaluate L-FABP binding to nuclei, the purified protein was conjugated with fluorescein (FLUOS-FABP). When rat liver nuclei were incubated with FLUOS-FABP in the presence of 0.5 mM oleic acid, a strong fluorescence response was observed in the nuclei (**Fig. 4**). A comparison of the fluorescent and light microscopic views demonstrates that the bright fluorescent images correspond precisely with the position of the nuclei (compare Fig. 4 right, top and bottom). In the absence of oleic acid, no fluorescence was associated with the nuclei (compare Fig. 4 left, top and bottom).

To quantify the binding of FLUOS-FABP to nuclei, we developed a fluorescent filter-binding assay. In the presence of 0.5 mM oleic acid, the association of FLUOS-FABP increased as a function of nuclear DNA (**Fig. 5A**). A plateau was achieved when the nuclear DNA content exceeded 0.6 μg. The association of FLUOS-FABP with the nuclei was dependent on the concentration of oleic acid and no binding was observed in the absence of fatty acid (Fig. 5B). The EC_{50} for oleic acid stimulation of FLUOS-FABP binding was 0.2 mM and saturation was achieved between 0.4 and 0.6 mM. These concentrations are high because of the presence of 0.2% fatty acid-free BSA, which was included to reduce nonspecific binding. The BSA will bind and sequester a large portion of the added oleic acid, thus increasing the concentration required to occupy the binding sites in L-FABP. Richieri, Anel, and Kleinfeld (48) demonstrated that the free concentrations of fatty acids in aqueous environments containing BSA were independent of the absolute fatty acid concentration, but dependent on the ratio of fatty acid to BSA. They reported that when the ratio of oleate to BSA was 3, the same as the ratio in our nuclear binding reaction when the oleate concentration is 0.1 mM, the free oleate concentration is estimated to be 20 nM. In addition, when the ratio of oleate to BSA is 5 the free concentration of oleate is estimated to be approximately 300 nM. Thus, the free oleate available to bind to FABP is considerably lower in the presence of BSA.

In the presence of 0.5 mM oleic acid, FLUOS-FABP binding to nuclei increased as a function of its concentra-

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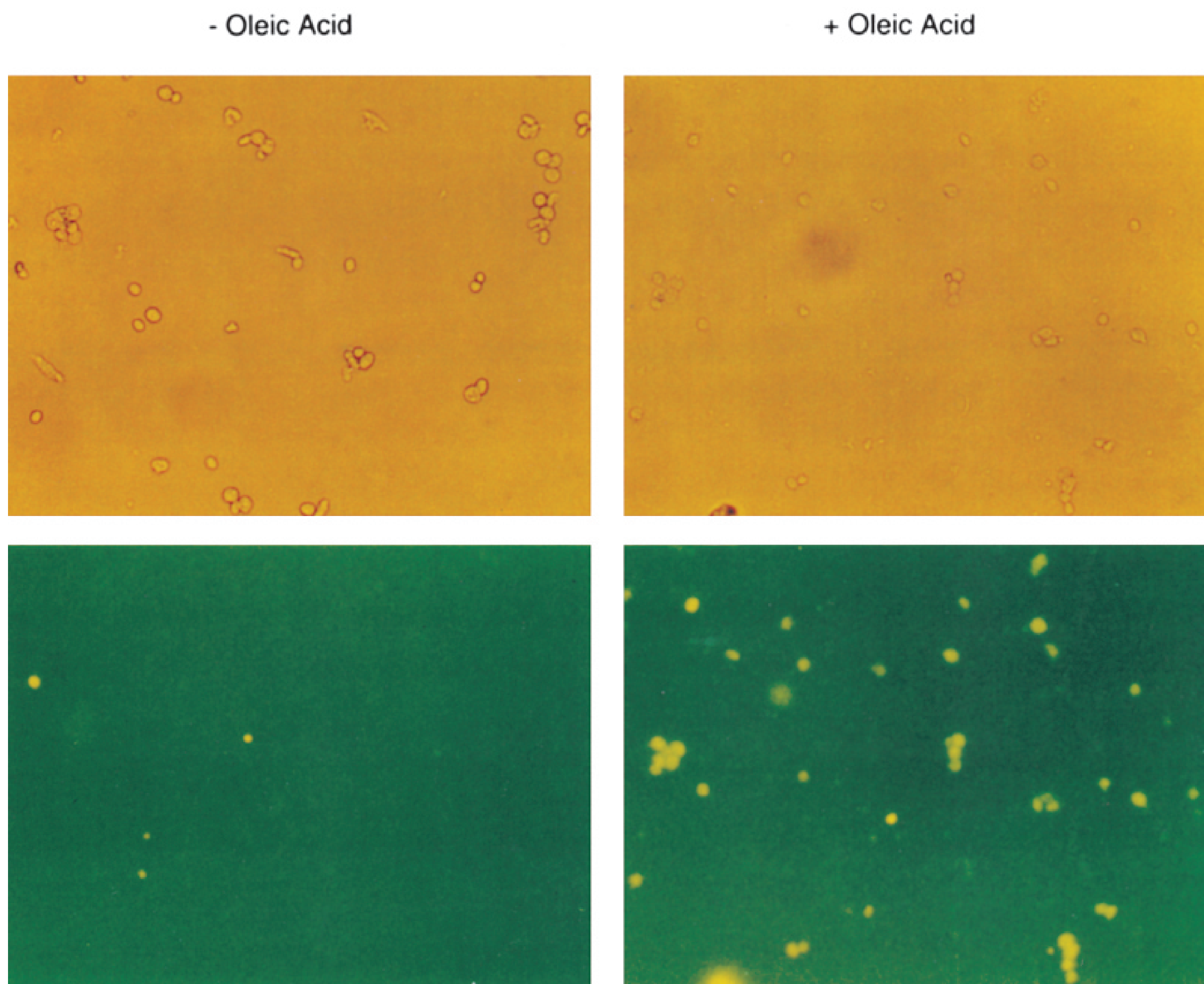

Fig. 4. Ligand-dependent localization of L-FABP to nuclei in situ. FLUOS-conjugated L-FABP (140 nmol) was incubated in the absence (left) or presence (right) of oleic acid (0.5 mM) in binding buffer containing 0.2% fatty acid-free BSA at 37°C for 10 min to allow binding of the fatty acid to the protein. Rat liver nuclei (3.3 μg of DNA) were then added and incubated at room temperature for 30 min. Identical fields of nuclei were viewed by light (top) and fluorescence (bottom) microscopy.

tion in the range 300–750 nM (Fig. 5C). The binding was saturated above 750 nM, suggesting a limited number of binding sites. The nuclear associated fluorescence was progressively reduced by the addition of increasing concentrations of native unlabeled L-FABP, with more than 80% inhibition at 19 μM unlabeled protein (Fig. 5D). The inhibitory effect was not due to sequestration of the fatty acid by native L-FABP, because increasing the concentration of oleic acid in the reaction to 1 mM did not prevent the competition (data not shown). Thus, the 0.5 mM oleate in the assay containing 0.2% BSA is sufficient to maintain all the L-FABP in the oleate-bound state.

Peroxisome proliferators facilitate L-FABP binding to nuclei

Several peroxisome proliferators including LY171883 (LY), bezafibrate, and WY-14,643 bind to L-FABP and displace oleic acid in a competitive manner (12). To determine if the compounds promote the interaction of L-FABP with nuclei, they were complexed to L-FABP and evaluated in [^{3}H]O:FABP competition experiments. LY:FABP caused a greater than 90% decrease in nuclear associated [^{3}H] O:FABP (**Fig. 6A**). The competition curve for LY:FABP was similar to that of O:FABP (Fig. 1A). L-FABP complexed to bezafibrate and WY-14,643 also inhibited the association of [^{3}H]O:FABP with nuclei (Fig. 6B). The magnitude of the inhibition was similar when L-FABP was complexed with an excess of nonradioactive oleic acid, LY171883, WY-14,643, or bezafibrate.

A peroxisome proliferator also promoted FLUOS-FABP binding to nuclei. In this experiment, the effect of LY189585 was compared with that of LY163443. LY189585 is a peroxisome proliferator in rats and ligand for L-FABP (12, 49). LY163443, a positional isomer of LY189585, is not a peroxisome proliferator and is a poor ligand for L-FABP. LY189585 caused a concentration-dependent association of FLUOS-FABP with nuclei (**Fig. 7**). Saturation was achieved at 4 mM and the EC_{50} was calculated to be 2 mM. On the ba-

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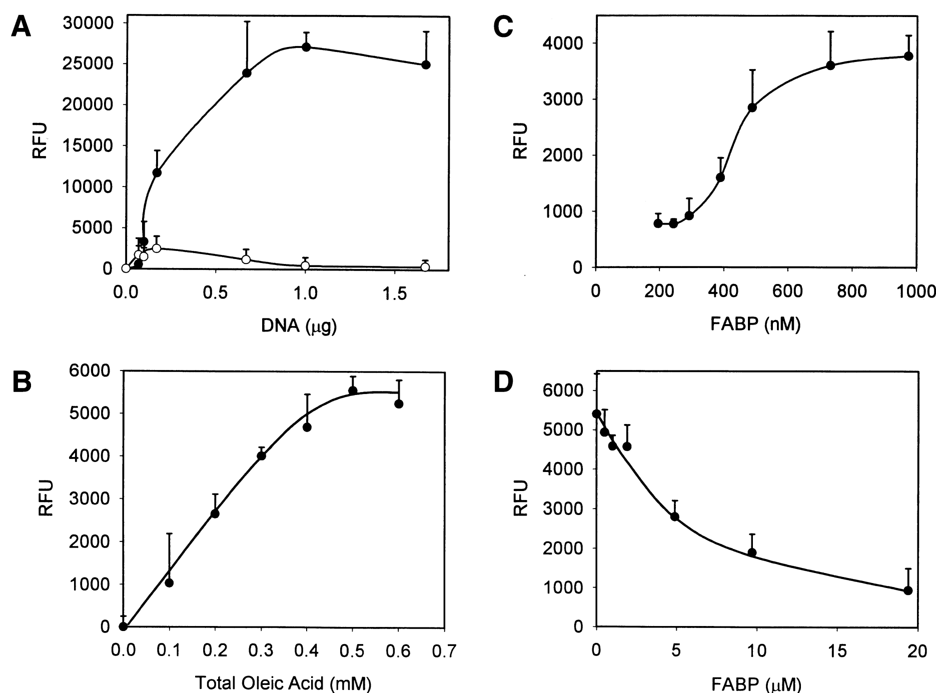

Fig. 5. Ligand-dependent binding of FLUOS-FABP to rat liver nuclei. FLUOS-FABP was incubated with oleic acid (when appropriate) for 10 min at 37°C in binding buffer containing 0.2% fatty acid-free BSA to allow binding of the fatty acid to the protein. Nuclei were then added and incubated at 25°C for 30 min. The nuclei were separated from unbound FLUOS-FABP, using a 96 well Fluoricon-GF assay plate and nuclear fluorescence quantified on a Pandex Screen Machine. (A) Increasing amounts of nuclei (DNA) were incubated with 140 pmol of FLUOS-FABP in the presence of either ethanol (open circles) or 0.5 mM oleic acid (solid circles). (B) Nuclei (3.3 μ of DNA) were incubated with 340 pmol of FLUOS-FABP and increasing concentrations of oleic acid. (C) Nuclei (1.7 μg of DNA) were incubated with increasing concentrations of FLUOS-FABP in the presence of 0.5 mM oleic acid. (D) Nuclei (2.3 μg of DNA) were incubated with 350 pmol of FLUOS-FABP and 0.5 mM oleic acid in the presence of increasing concentrations of unlabeled L-FABP. Values are expressed as the average ± standard error of three determinations.

sis of the findings of Richieri, Anel, and Kleinfeld for oleate (48), the actual free concentrations of LY171883 available for FABP are likely to be considerably lower because of the presence of BSA in the reaction buffer. LY163443 produced only a modest increase in nuclear binding. All concentrations of LY163443 induced the same amount of nuclear associated FLUOS-FABP, which was nearly 4-fold lower than that achieved with 4 mM LY189585.

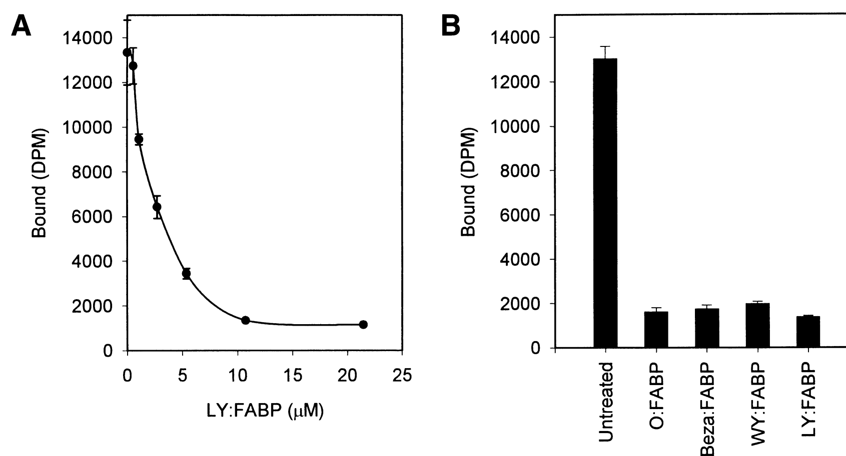

Fig. 6. Competition for nuclear binding of [^{3}H]oleic acid:L-FABP by unlabeled oleic acid or peroxisome proliferators complexed with L-FABP. Rat liver nuclei (2 μg of DNA) were incubated at room temperature for 50 min with 4 μM [^{3}H]O:FABP and (A) increasing concentrations of LY171883:FABP or (B) 18 μM unlabeled O:FABP, bezafibrate:FABP, WY-14,643:FABP, or LY171883:FABP. The binding was determined by filtration as described in Materials and Methods. Values are expressed as the average ± standard error of three determinations.

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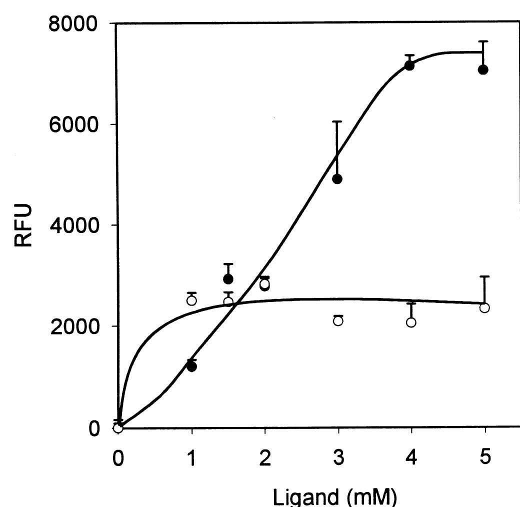

Fig. 7. Comparison of a peroxisome proliferator (LY189585) and a nonperoxisome proliferator (LY163443) in the FLUOS-FABP nuclear binding assay. FLUOS-FABP (55 pmol) and increasing concentrations of LY189585 (solid circles) or LY163443 (open circles) were incubated for 10 min at 37°C to allow binding of the ligand to the protein. Nuclei (3.3 μg of DNA) were then added and incubated at 25°C for 30 min. The nuclei were separated from unbound FLUOS-FABP, using a 96-well Fluoricon-GF assay plate and nuclear fluorescence was quantified on a Pandex Screen Machine. Values are expressed as the average ± standard error of three determinations.

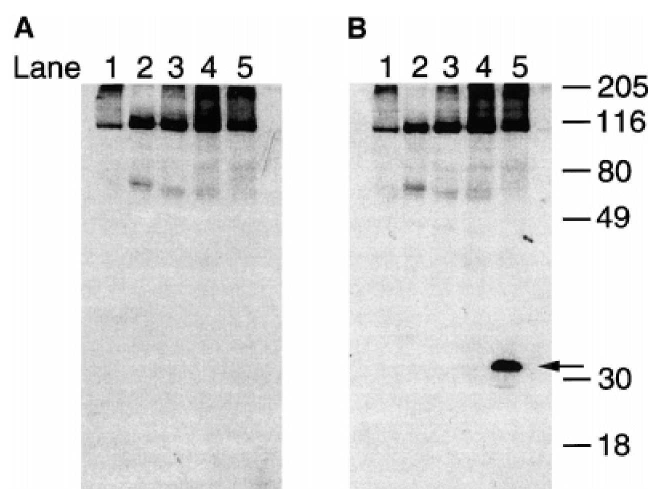

Fig. 8. Interaction of L-FABP with nuclear proteins. The binding of biotinylated L-FABP to nuclear proteins was analyzed by Far Western analysis (46). Proteins from rat liver postnuclear supernatants (lane 1), the nuclear wash (lane 2), and sequential 150, 300, and 500 mM NaCl nuclear extracts (lanes 3–5) were resolved through 12% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were blocked with TNE-50 buffer containing 2% fatty acid-free BSA and incubated at 37°C for 2.5 h in the absence (A) or presence (B) of biotinylated L-FABP (200 ng/ml). After washing, the blots were developed with avidin-HRP and the binding was determined as described in Materials and Methods.

L-FABP binds to nuclear proteins in a fatty acid-dependent manner

The observation that the binding of [^{3}H]O:FABP to rat liver nuclei was abolished by proteinase treatment suggested a protein-protein interaction. This possibility was evaluated by Far Western analysis, which was conducted by incubating biotin-conjugated L-FABP (bFABP) with nuclear proteins that were separated by gel electrophoresis and immobilized on nitrocellulose. Biotinylated L-FABP was detected by avidin-horseradish peroxidase (HRP)-dependent chemiluminesence. In control incubations lacking bFABP, high molecular weight proteins interacted with avidin-HRP, probably representing endogenous biotin-containing proteins (**Fig. 8A**). When the protein blot was incubated with bFABP prior to development with the avidin-HRP, a 33-kDa band appeared that did not appear in the control blot (Fig. 8B). This protein was found in the 500 mM NaCl extract of hepatocyte nuclei but not in 150 and 300 mM NaCl nuclear extracts, the postnuclear supernatants, or a nuclear wash. The same protein was identified in Far Western blots using ^{125}I-labeled L-FABP or fluorescein-conjugated L-FABP, suggesting that the interaction with the 33-kDa protein was not dependent on the method of labeling L-FABP (data not shown). The interaction of bFABP with the 33-kDa protein was decreased greater than 80% by the addition of excess unlabeled L-FABP to the incubation (**Fig. 9**).

The interaction demonstrated in Figs. 8 and 9 occurred in the absence of added fatty acid. The L-FABP-nuclear protein interaction was further evaluated in the absence or presence of 0.5 mM oleic acid. The blots contained 500 mM NaCl extract that was fractionated with a 100,000-kDa cutoff ultrafiltration unit to remove the high molecular weight avidin-binding proteins. The binding of bFABP to the 33-kDa protein in the absence of fatty acid increased as a function of nuclear extract on the blot (**Fig. 10A**). Two additional bands of approximately 30 and 26 kDa were also observed at the higher concentrations of protein tested. In the presence of 0.5 mM oleic acid, the interaction of bFABP with the proteins on the blot was markedly enhanced (Fig. 10B). In this case, several other protein bands appeared, suggesting there may be several nuclear proteins that interact with L-FABP.

DISCUSSION

The demonstration that fatty acids regulate hepatic gene expression has raised the question of how the relatively insoluble fatty acids are transported to the nucleus. FABP is a likely candidate for this function based on its role in transporting fatty acids between other cellular compartments (1–4). Our initial approach to explore the role of L-FABP in fatty acid transport to the nucleus was modeled after studies that demonstrated a role for the cellular retinol-binding protein (CRBP) in the nuclear transport of retinol (34, 35). We were able to demonstrate only nonspecific binding of free [^{3}H]oleic acid to rat liver nuclei in the absence of FABP, using a variety of techniques and assay conditions. However, specific binding to nuclei was observed when the [^{3}H]oleic acid was complexed to FABP.

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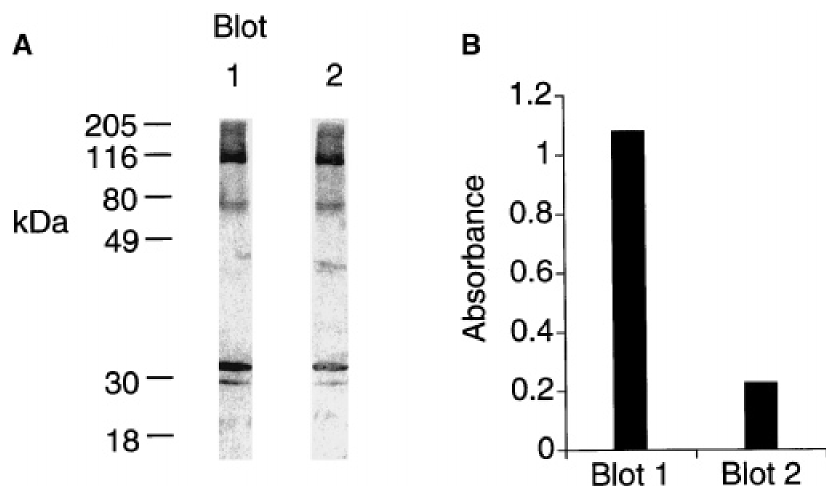

Fig. 9. Inhibition of biotinylated L-FABP binding to nuclear proteins by native L-FABP. Proteins in the 500 mM NaCl nuclear extract were separated by SDS-polyacrylamide gel electrophoresis and immobilized on nitrocellulose membranes. (A) Far Western analysis was conducted with biotinylated L-FABP (200 ng/ml) in the absence (blot 1) or presence (blot 2) of a 1,000-fold excess of unlabeled L-FABP. (B) Densitometric analysis of 33-kDa band on lumigram.

A technical issue regarding the fact that the oleate is not covalently linked to the FABP arises during these studies. Redistribution of the oleate molecules between FABP and BSA could occur. We have demonstrated that fatty acid-free BSA will act as an acceptor of the tritiated oleate complexed to FABP, simulating competition; however, when the fatty acid-binding sites are already filled with a fatty acid (oleate in this study), no exchange occurs. Exchange of the tritiated oleate from FABP probably happens with fatty acid-free FABP as well; however, this will not appear as competition. This can be explained by the fact that the exchange reaction results in the production of one molecule of fatty acid-free FABP and one molecule of tritiated oleate-bound FABP, exactly the same as the starting reaction components. Thus, no competition occurs if the fatty acid-free FABP has low or no affinity for the nuclear binding sites. This concept is supported by the FLUOS-FABP-binding studies that demonstrated that nuclear binding occurs only in the presence of ligand. These data suggest that fatty acids can exchange from one protein to another in an aqueous environment; however, our data also suggest that little exchange occurs when no open high-affinity acceptor sites are present in an aqueous environments, similar to our binding conditions. In addition, the inability of added fatty acid-free BSA to facilitate the association of oleate with the nucleus demonstrates that the function of FABP is unique and makes it unlikely that it facilitated nuclear binding simply by increasing the solubility of oleate.

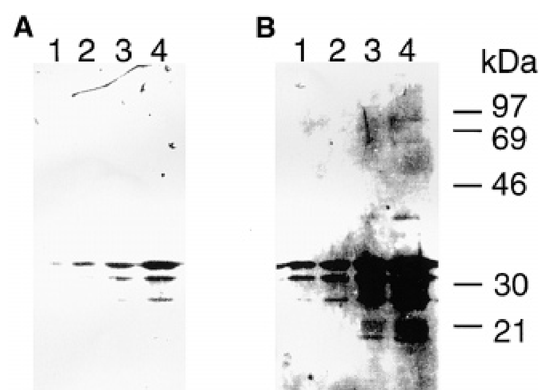

Fig. 10. Binding of biotinylated L-FABP to nuclear proteins in the presence of oleic acid. Increasing amounts of 500 mM NaCl extract of rat liver nuclei were separated by SDS-polyacrylamide gel electrophoresis and immobilized on nitrocellulose membranes: 5 μg of extract (lane 1), 10 μg of extract (lane 2), 25 μg of extract (lane 3), 50 μg of extract (lane 4). Binding of biotinylated L-FABP to nuclear proteins was determined by Far Western analysis in the absence (A) or presence (B) of 0.5 mM oleic acid in TNE-50 buffer containing 0.2% fatty acid free BSA.

The experiments in which FABP was labeled with fluorescein demonstrated that the protein interacted directly with the nuclei. The nuclear interaction of FABP was dependent on the presence of oleate, which is consistent with the studies using [^{3}H]O:FABP. Clearly, oleate alters a property of FABP that converts it to a specific ligand for nuclear binding. The binding of oleate causes a conformational change in liver FABP, which has been demonstrated by limited protease digestion, circular dichroism, and nuclear magnetic resonance (50, 51). The ligand-induced conformational change may be a characteristic of this family of intracellular binding proteins in that both heart FABP and cellular retinol-binding protein undergo such changes (52). The functional importance of the conformational changes has been demonstrated in the case of nuclear hormone receptors including estrogen receptor and PPAR. The transcriptional functions of these receptors are activated by a ligand-induced conformational change (53–55). It is feasible that oleate-induced changes in the conformation of FABP facilitate its interaction with organelles that utilize the fatty acid.

Amphiphilic compounds that induce peroxisome proliferation were found previously to bind to L-FABP and displace oleic acid (12). It seemed likely to us that peroxisome proliferator-bound L-FABP would interact with nuclei similar to oleate-bound L-FABP. In the case of each compound tested, the peroxisome proliferator-FABP complex competed with [^{3}H]O:FABP for binding to nuclei. The peroxisome proliferator LY171883 was as effective as oleate at inhibiting the binding when the compounds were

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complexed with FABP at saturating concentrations (10 μM oleate and 500 μM LY171883). Thus, oleate and LY171883 confer the same property on FABP, although LY171883 binds with a 10-fold lower affinity (12). Similar observations were made previously with clofibric acid. This peroxisome proliferator caused a conformational change in FABP similar to that induced by oleate despite having a far lower affinity (50). LY171883 and clofibric acid induce indistinguishable conformational changes in mouse PPARα although their potencies differ (56). Likewise, conformational changes in PPARγ were induced by a saturating concentration of agonists whose affinities differed by approximately 100-fold (53). Therefore, the differences in the affinity of oleate and LY171883 do not preclude the possibility that they induce a similar conformational change in FABP, resulting in its interaction with the nucleus. In contrast, an inability to bind to FABP should preclude a compound from facilitating the interaction of FABP with the nucleus. The effects of LY189585 and LY163443 on FLUOS-FABP binding are consistent with their affinities for FABP. The affinity of LY189585 for FABP is approximately 20-fold lower than oleate. The oleate concentration required for maximum binding of FLUOS-FABP to nuclei was approximately 0.4 mM. The concentration of LY189585 required for maximum binding was approximately 10-fold higher (4 mM), consistent with its weaker affinity. Moreover, the affinity of LY163443 for FABP is approxiamtely 5–10 times weaker than LY189585, consistent with its lack of dose response and minimal effect on FLUOS-FABP nuclear binding. In addition, the findings of Richieri, Anel, and Kleinfeld (48) suggest the actual free concentrations of LY189585 and LY163443 available for FABP are likely to be considerably lower because of the presence of BSA in the reaction buffer. Thus, using both [^{3}H]oleate and a fluorescent label to track FABP, we were able to demonstrate that peroxisome proliferators facilitate the interaction of FABP with the nucleus in a manner similar to oleate. We have proposed that peroxisome proliferators such as LY171883, LY189585, clofibric acid, and WY-14,643 share structural properties with fatty acids that allow them to bind to FABP (57). It is likely that this binding results in a conformational change in L-FABP that facilitates its interaction with the nucleus.

We considered the possibility that hepatic FABP associates with nuclei through interactions with DNA based on the proposal by other investigators that the helix-turn-helix motif in cardiac FABP has a role in its localization to the nucleus (47). We found that the digestion of DNA by nuclease treatment did not impair the association of [^{3}H]O:FABP with nuclei. In contrast, proteinase digestion produced a marked inhibition of [^{3}H]O:FABP binding to nuclei, suggesting that protein interactions facilitated the binding. Far Western analysis confirmed the presence of FABP-interacting proteins in rat liver nuclear extracts. The most prominent was a 33-kDa protein found in the 500 mM NaCl extract of nuclei. Its absence in the less stringent nuclear extracts suggests that the 33-kDa protein is a strongly associated nuclear protein rather than a cytosolic protein carried over in the preparation of the nuclei. The interaction of FABP with the 33-kDa protein was detected in the absence of fatty acid; however, the interaction was increased considerably in the presence of oleic acid. The differences in the level of binding in the absence and presence of oleic acid were most notable with smaller quantities of protein on the blot. Thus, in a fashion similar to the fluorescence binding assay, optimum binding of biotinylated FABP to the nuclear protein is observed in the presence of ligand. FABP interacted with several proteins in addition to the 33-kDa protein in the presence of oleic acid. The data suggest that the association of L-FABP with the nucleus may occur through its interaction with specific proteins, such as those detected in the Far Western analysis.

The conformational change that FABP undergoes in response to ligand could provide a mechanism to ensure a supply of fatty acid to the nucleus. L-FABP has a molecular mass of 14 kDa, which would allow it to freely diffuse into the nucleus. Our data suggest that apo-FABP has poor affinity for the nucleus whereas ligand-bound FABP has a greater affinity for the nuclear binding site and localizes in the nucleus. On transfer of ligand, FABP is proposed to revert to the conformation with low affinity for the nuclear binding site and diffuse back into the cytosol. The concentration of FABP in the liver cell is approximately 200–400 μM (58, 59), accounting for up to 5% of the cytoplasmic protein (6). However, only 2% of the L-FABP is in the ligand-bound state (58–60). Therefore, the actual quantity of FABP available for interaction with the nucleus is low in the normal rat liver. We propose that as the concentration of fatty acid or other ligand increases in the hepatocyte, the FABP-ligand complex will accumulate in the nucleus. In the case of peroxisome proliferators, the ligand-bound FABP may be more persistent because these chemicals are more resistant to metabolism than fatty acids.

The fate of the fatty acid after the FABP-ligand complex is transported to the nucleus could include one of several possibilities. FABP may deliver the fatty acid to other nuclear proteins, including the ligand-dependent transcription factors, or the FABP-fatty acid complex may directly interact with transcriptional components in the nucleus. Another possibility is that FABP may provide a specific pool of fatty acids for nuclear utilization. Fatty acids are known to modify the activity of certain nuclear enzymes, including DNA nucleotidase and DNA-dependent RNA polymerase (61). In addition, the nucleus contains enzymes that metabolize fatty acids, including long-chain fatty acyl-CoA synthase (62) and Δ^5-desaturase, which is involved in arachidonate synthesis (63, 64). Hence, the role of FABP might be to deliver substrates to the nucleus for metabolic conversion.

In conclusion, we have demonstrated that hepatic FABP interacts with rat liver nuclei in a specific manner in the presence of oleic acid. Several peroxisome proliferators can substitute for fatty acid to facilitate the nuclear interaction. In addition, hepatic FABP interacts with several rat liver nuclear proteins in the presence of ligand, suggesting that these may be the nuclear docking proteins for ligand-bound L-FABP. Currently, we are trying to deter-

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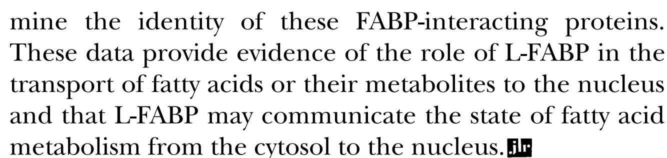

mine the identity of these FABP-interacting proteins. These data provide evidence of the role of L-FABP in the transport of fatty acids or their metabolites to the nucleus and that L-FABP may communicate the state of fatty acid metabolism from the cytosol to the nucleus.

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