Bovine Epidermal Fatty Acid-Binding Protein: Determination of Ligand Specificity and Cellular Localization in Retina and Testis†

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ABSTRACT: The fatty acid-binding protein (FABP) family consists of small, cytosolic proteins believed to be involved in the uptake, transport, and solubilization of their hydrophobic ligands. Members of this family have highly conserved sequences and tertiary structures. Using an antibody against testis lipid-binding protein, a member of the FABP family, a protein was identified from bovine retina and testis that coeluted with exogenously added docosahexaenoic acid during purification. Amino acid sequencing and subsequent isolation of its cDNA revealed it to be nearly identical to a bovine protein expressed in the differentiating lens and to be the likely bovine homologue of the human epidermal fatty acid-binding protein (E-FABP). From quantitative Western blot analysis, it was estimated that bovine E-FABP comprised 0.9%, 0.1%, and 2.4% of retina, testis, and lens cytosolic proteins, respectively. Binding studies using the fluorescent probe ADIFAB indicated that this protein bound fatty acids of differing levels of saturation with relatively high affinities. K_d values ranged from 27 to 97 nM. In addition, the protein was immunolocalized to the Müller cells in the retina as well as to Sertoli cells in the testis. The location of bovine E-FABP in cells known to be supportive to other cell types in their tissues and the ability of E-FABP to bind a variety of fatty acids with similar affinities indicate that it may be involved in the uptake and transport of fatty acids essential for the nourishment of the surrounding cell types.

The transport and solubilization of fatty acids and retinoids are facilitated by a family of proteins known as intracellular lipid-binding proteins. Members of this family include the fatty acid-binding proteins (FABPs)[1] and the four cellular retinoid-binding proteins. Members of the family are approximately 14−15 kDa in size and share a high degree of sequence conservation across species as well as among family members (*1*−*3*). The tertiary structure, for all proteins solved, is also remarkably conserved. Ten antiparallel β-strands form a flattened barrel with a hydrophobic pocket in the center that accommodates hydrophobic ligands (*2*).

Although their physiological functions are not known with certainty, it is thought that FABPs are involved in the uptake, transport, and metabolism of their ligands (*1, 2*). There is also evidence to suggest that they may have an influence, indirectly, on cell signaling and regulation of gene expression through the peroxisome proliferator-activated receptor or the fatty acid-activated receptor (*1, 4*). The distinct tissue distributions, cellular localizations, binding affinities, and often stage-specific expression patterns of these proteins imply that each member has a unique role to play in the cell type in which it is located.

We were interested in identifying a binding protein that might be involved in the transport of docosahexaenoic acid (DHA; C22:6), a long-chain polyunsaturated fatty acid of the ω3 family that is synthesized from the essential fatty acid linolenic acid (C18:3ω3). Earlier work has suggested that binding protein(s) for DHA and other fatty acids exist(s) in the retina (*5, 6*), where DHA is highly abundant in membrane phosphatidylcholines.

Our approach was to isolate proteins from bovine retina and testis that could cross-react with antibodies against testis lipid-binding protein (TLBP), another family member, and coelute with exogenously added DHA. We describe here the isolation of a single protein from both tissue sources that is the likely bovine homologue of human epidermal FABP (E-FABP). Ligand-binding studies revealed that it bound fatty acids of varying degrees of saturation with relatively high affinities (i.e., in the nanomolar range). We have also localized the protein to the retinal Müller cells and to Sertoli cells in the testis. On the basis of these results, we propose that E-FABP is involved in the metabolism/delivery of fatty acids essential to neighboring cells.

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[1] Abbreviations: FABP, fatty acid-binding protein; E-FABP, epidermal FABP; H-FABP, heart FABP; I-FABP, intestinal FABP; ALBP, adipocyte lipid-binding protein; BLBP, brain lipid-binding protein; KLBP, keratinocyte lipid-binding protein; TLBP, testis lipid-binding protein; CRBP, cellular retinol-binding protein; ADIFAB, acrylodated I-FABP; DHA, docosahexaenoic acid; KLH, keyhole limpet hemocyanin; PMSF, phenylmethanesulfonyl fluoride.

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EXPERIMENTAL PROCEDURES

Materials. Bovine tissues were obtained from a local slaughterhouse except for frozen bovine testes used in protein isolation which were from Pel-Freez Biologicals. Male New Zealand rabbits used in antibody production were from Myrtle's Rabbitry and were housed in the Vanderbilt University Animal Care Facility. (Studies involving animals were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the oversight of veterinarians and our local Institutional Animal Care and Use Committee.) TiterMax was from CytRx Corp. Keyhole limpet hemocyanin (KLH) was from Pierce. DTT and protease inhibitor cocktail tablets were from Boehringer Mannheim. Phenylmethanesulfonyl fluoride (PMSF), pepstatin, aprotinin, leupeptin, and *all-trans*-retinol were from Sigma. Sephacryl S-100, Sephadex G-75, and Sephadex G-50 resins were from Pharmacia. DE-52 resin was from Whatman. [^{3}H]DHA was from DuPont NEN (60 Ci/mmol). The sodium salts of fatty acids used in ligand-binding studies were from Nu Chek Prep. ADIFAB was from Molecular Probes. The bovine retina cDNA library was from Stratagene. [α-^{32}P]dCTP was from ICN (3000 μCi/mmol). Goat anti-rabbit IgG and 18 nm colloidal gold AffiniPure goat anti-rabbit IgG were from Jackson ImmunoResearch. Lowicryl K4M and K11M were from Polysciences. Murine KLBP and rat I-FABP were gifts from Dr. Judith Storch (Rutgers University). All other materials were of reagent grade or better.

Preparation of Cytosolic Fractions. Retinas were stripped at 4 °C in minimal PBS with 0.02% EDTA, snap frozen on dry ice, and stored at −70 °C. Frozen tissues were thawed in 0.9% cold saline and homogenized with a Polytron homogenizer at 14 000 rpm for 2 × 30 s or a Waring blender in 3× volume of PBS containing either 5 μM pepstatin, 5 μg/μL aprotinin, 1 mM PMSF, 5 μM leupeptin, and 0.2 mM EDTA or a protease inhibitor cocktail tablet. The homogenates were centrifuged for 20 min at 20000*g*, and the supernatant was centrifuged 60 min at 100000*g*. The protein concentration of the supernatant was determined by BCA protein analysis (Pierce), and aliquots were stored at −70 °C.

Protein Isolation from Tissue. The basic protocol for protein separation was as follows. Cytosol was concentrated 2−3-fold in an Amicon stirred cell with a YM3 (3000 MW cutoff) membrane before being equilibrated with a small amount of retinol in dimethyl sulfoxide. Fractionation was performed on a 0.75 L Sephacryl S-100 column or a 1.5 L Sephadex G-75 column equilibrated with buffer A [20 mM Tris−acetate (pH 8.3 at 25 °C)] with 1 mM DTT. Fractions were collected and analyzed to determine the location of the 15 kDa proteins by fluorescent emission of CRBP-bound retinol at 490 nm after excitation at 348 nm with an SLM-Aminco SPF-500C spectrofluorometer. Fractions were also analyzed on Western blot as previously described (*7*) using Amersham's ECL detection system and the previously isolated polyclonal anti-TLBP IgG at a dilution of 1/100 (*8*). Immunoreactive fractions of approximately 15 kDa were pooled and concentrated in an Amicon stirred cell with a YM3 membrane.

[^{3}H]DHA (5 μmol) was added to the pooled protein (from testis) and allowed to equilibrate at 4 °C before being loaded onto a 20 mL DE-52 column equilibrated in buffer A. Protein was eluted with a 100 mL linear gradient of 20 mM Tris−acetate (pH 8.3) to 330 mM Tris−acetate (pH 8.3). Fractions were analyzed by SDS−PAGE on the PhastSystem (Pharmacia Biotech, Inc.) and by Western blot using affinity-purified anti-TLBP IgG at a 1/100 dilution. The elution of [^{3}H]DHA was determined by measuring the radioactivity of selected fractions.

Amino Acid Sequence Determination. Fractions that eluted with [^{3}H]DHA and/or contained a 15 kDa immunoreactive protein were resolved on a 15% SDS−polyacrylamide gel and electrophoretically transferred to a PVDF membrane (Bio-Rad) as described previously (*7*). The membranes were cut in half and used for Western blot analysis, or the proteins were visualized by Coomassie staining. Coomassie-stained bands corresponding to the immunoreactive protein were subjected to internal automated Edman degradation (Applied Biosystems).

Isolation of cDNA Clone. Degenerate oligonucleotide primers were designed on the basis of amino acid sequencing and prepared on an Applied Biosystems DNA synthesizer. The sequences of the primers were 5′-GAYGAR-TAYATGAARGARGT-3′ and 5′-CCRTCCCAYTCYT-GRTG YTG-3′ for the sense and antisense strands, respectively. Thirty cycles of PCR with an annealing temperature of 55 °C were performed in a Robocycler (Stratagene) using the degenerate primers and boiled bovine retina cDNA library. The resulting PCR product was gel-extracted (Qiagen kit) and labeled with [α-^{32}P]dCTP using the Redi-Prime kit (Amersham). Bovine retina cDNA library screening using this labeled product was performed according to supplier guidelines. Positive clones were sequenced and subsequently analyzed using MacVector and Entrez programs.

Expression and Purification of E-FABP in Escherichia coli. The coding sequence of E-FABP was cloned into the prokaryotic expression vector pRSET after the introduction of an *Nde*I site 5′ and a *Hin*dIII site 3′ to the coding sequence by PCR.

Following transformation, protein expression was induced in log phase BL21(DE3)/pLysS cells with 1 mM isopropyl thio-β-D-galactoside for 3.5 h at 37 °C. Cells were pelleted, resuspended in Buffer B [10 mM Tris−acetate (pH 8.3 at room temperature) and 1 mM DTT] supplemented with a protease inhibitor cocktail tablet, and sonicated to lyse. The lysate was centrifuged 30 min at 30000*g*. To improve protein stability, the supernatant was incubated with 0.1 mM sodium oleate before being fractionated on an 800 mL Sephadex G-50 column equilibrated with buffer B. Proteins retained in the inclusion volume were pooled, and the volume was reduced to 30 mL in an Amicon stirred cell. Additional sodium oleate and glycerol (10% final concentration) were added. The protein sample was applied to an 18 mL DE-52 column in buffer B with 10% glycerol and eluted in the break-through fractions. Purified protein was delipidated on a Lipidex column as previously described (*9*) and appeared as a single band on a silver-stained gel (Figure 1c). Protein concentration was determined using the calculated molar extinction coefficient of 13 940.

Preparation of Immune Reagents and Immunolocalization. A peptide with the sequence 5′-TVQQLVGRWRLVESKGC-3′, corresponding to residues 3−18 plus an additional

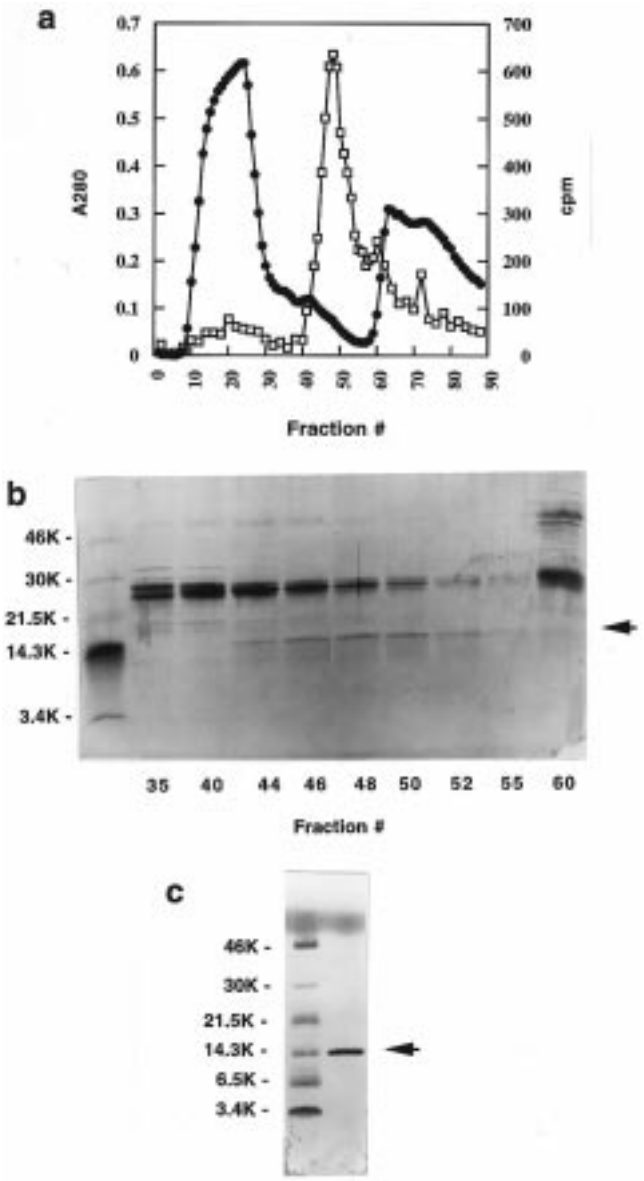

FIGURE 1: Isolation of E-FABP from bovine testis and purification of recombinant E-FABP. Testis cytosol (●) equilibrated with [^{3}H]-DHA (□) was fractionated on an anion-exchange resin with a linear Tris−acetate gradient (a). A silver-stained gel of the eluted fractions revealed that a protein of approximately 15 kDa (indicated by the arrow) was present in the same fractions, corresponding to the largest [^{3}H]DHA peak (b). (c) Silver-stained gel of purified, recombinant E-FABP. Approximately 55 ng of E-FABP, purified as described in Experimental Procedures, was loaded. A single band (at the arrow) is present in the right lane; molecular weight markers are in the left lane.

cysteine residue, was coupled to the carrier protein KLH. An emulsion of the coupled peptide with the adjuvant TiterMax was injected into two rabbits intradermally on their backs. The rabbits were boosted with coupled peptide intradermally and intramuscularly after 5 weeks and with recombinant glutaraldehyde cross-linked protein after an additional 5 weeks. The IgG fraction was isolated from serum using a protein A column. Antibodies specific for the recombinant protein (1497αFABP) were purified by passing the IgG fraction over a SulfoLink column (Pierce) containing coupled recombinant protein according to supplier's protocol. Flow-through and eluted fractions from the affinity column were aliquoted and stored at −70 °C.

Bovine testis was fixed within 20 min post mortem. The fixation and staining of tissues with a biotin and alkaline phosphatase detection system are described elsewhere (*7*). Affinity-purified IgG (0.5 OD) was used at 1/1000 (retina) or 1/100 (testis) dilutions for immunolocalization.

Electron Microscopic Immunocytochemistry. Following removal of the cornea, iris, lens, and vitreous from bovine eyes, the resulting eyecup was fixed by immersion for 2 h in 4% formaldehyde and 0.5% glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.2. The choroid and retina were dissected from the sclera, dehydrated stepwise in methanol at −20 °C, and infiltrated and embedded in either Lowicryl K4M or Lowicryl K11M. Fragments of tissue were placed in flat molds, and the molds were then filled with resin and covered with acrylic sheets. The resins were maintained at −20 °C, polymerized overnight with long-wavelength ultraviolet (UV) light, and subsequently exposed to UV light at room temperature for an additional 2 days.

Ultrathin sections were cut with glass knives, placed on Formvar carbon-coated grids, and stained with 1497αFABP IgG at a concentration of 0.1 OD (A_{280}, 1 cm light path) for 1 h at room temperature. Binding sites for the primary antibodies were revealed by staining for 1 h with 18 nm colloidal gold adsorbed to affinity-purified goat anti-rabbit IgG (Jackson ImmunoResearch). Tissue contrast was enhanced with 5% aqueous uranyl acetate.

Ligand-Binding Studies. The fluorescent probe ADIFAB was used in a ligand-binding assay as previously described (*10, 11*). Briefly, aliquots of fatty acid sodium salts were titrated into a cuvette containing ADIFAB (0.2 μM) and bovine E-FABP (4 μM). Fluorescent emission at 432 and 505 nm after excitation at 386 nm was monitored for each addition with an SLM Aminco 8100 spectrofluorometer. The amount of free fatty acid in equilibrium with ADIFAB and E-FABP as well as fatty acid bound to ADIFAB was determined for each addition. From those values and the previously published K_d values for ADIFAB alone with fatty acids (*12*), the amount of fatty acid bound to E-FABP could be calculated. Data were plotted in a Scatchard plot for analysis.

RESULTS

The polyunsaturated long-chain fatty acid DHA (C22:6ω3) is highly abundant in tissues that require increased membrane fluidity. Its presence in membranes is essential for sperm motility (*13*−*15*), for the proper functioning of rhodopsin in the retina (*16*−*21*), and for neuron function (*22, 23*). To identify a protein that might be involved in the binding and trafficking of DHA, two tissues known to be abundant in DHA, testis and retina, were examined. Protein was isolated from each tissue by a combination of gel filtration and anion-exchange chromatography. The progress of the separation was monitored by cross-reaction with antibodies against TLBP, another member of the FABP family, and by coelution with DHA added to the protein sample. From this fractionation procedure, an immunoreactive protein of approximately 15 kDa was identified in both tissues (Figures 1 and 4b). Densitometry of the 15 kDa band indicated that its abundance correlated with the elution peak of radiolabeled DHA.

Proteins from both sources were subjected to internal amino acid sequencing. Five peptide fragments from the

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5′        GCACGAGGCGCCCTACCCTCCGCAGCTCACCTGTCACGCCTGTCCGCTCGGACCCACC

ATGGCCACCGTTCAGCAGCTGGTAGGAAGATGGCGCTTAGTGGAGAGCAAAGGATTTGACGAATAC
M  A  T  V  Q  Q  L  V  G  R  W  R  L  V  E  S  K  G  F  D  E  Y
1                                                               22

ATGAAGGAAGTAGGAGTGGGGATGGCTCTGCGAAAAGTGGGTGCGATGGCCAAACCAGACTGTATC
M  K  E  V  G  V  G  M  A  L  R  K  V  G  A  M  A  K  P  D  C  I
23                                                              44

ATCACTTCTGATGGCAAAAACCTCAGCATAAAGACTGAGAGCACTTTGAAAACAACACAGTTTTCC
I  T  S  D  G  K  N  L  S  I  K  T  E  S  T  L  K  T  T  Q  F  S
45                                                              66

TGTAAACTGGGAGAGAAGTTTGAAGAGACCACAGCTGATGGCAGAAAGACTCAGACTGTCTGCAAC
C  K  L  G  E  K  F  E  E  T  T  A  D  G  R  K  T  Q  T  V  C  N
67                                                              88

TTTACAGATGGCGCATTGGTTCAACATCAGGAATGGGATGGAAAGGAAAGCACAATAACAAGAAAA
F  T  D  G  A  L  V  Q  H  Q  E  W  D  G  K  E  S  T  I  T  R  K
89                                                             110

CTGGAAGATGGGAAATTAGTGGTGGTATGCGTCATGAACAATGTTACCTGTACTCGGGTCTATGAA
L  E  D  G  K  L  V  V  V  C  V  M  N  N  V  T  C  T  R  V  Y  E
111                                                            132

AAAGTAGAGTAAAAATTCCCTCATCATTTTGCACAGAAATTAGCTGCGAGGATGAACAAGCTCAGT
K  V  E  *
133

TCAATGAGCAAATCTCCATACTGCTGGTTTTTTTTTTTCATTATTGCGTTCAATTATCTTTATCAC

AAACAAACTATTTCAAAGTGTTGGTTTAATTAGGGTCATCTCTTTGGTTAGTAAATAAATCTGTTT

GTGCTAAAAAAAAAAAAAAAAAAAA        3′
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FIGURE 2: Nucleotide sequence of the sense strand of E-FABP cDNA. The deduced amino acid sequence is indicated below. Residues identified by amino acid sequencing from retina (underlined) and testis (double-underlined) are also indicated.

retina protein were sequenced, representing about 41% of the final amino acid sequence. Two peptide fragments from the testis protein also were sequenced. Both fragments overlapped with peptides from the retina protein and were sufficiently long (14 and 17 residues) to indicate that the proteins from both sources were the same (denoted in Figure 2).

Knowledge of the partial amino acid sequence allowed the generation of a probe by PCR using degenerate primers. Subsequent screening of a bovine retina cDNA library identified a 677 bp clone with an open reading frame of 405 bp (Figure 2). The deduced amino acid sequence had 99% identity to a bovine lens protein (LP2) (*24*), 92% identity to human E-FABP (*25*, *26*), 76% identity to murine keratinocyte lipid-binding protein (KLBP) (*27*), 56% identity to bovine adipocyte lipid-binding protein (ALBP) (*28*), and 44% identity to rat TLBP (*8, 29*), placing this protein in the FABP family. Six nucleotide changes were observed between the previously reported bovine LP2 sequence and the sequence reported here (*24*). Four changes were in the noncoding region, and two were in the coding region, one of which indicated a different amino acid. The different amino acid predicted by our nucleotide sequence (Leu 52) was confirmed by amino acid sequencing. Despite these differences, it is likely that the two determined sequences represent the same protein. It is unusual, however, that while LP2 transcripts were detected in bovine lens, it was reported that no transcript was detected in retina (*24*). The degree of identity between human E-FABP and the bovine sequence reported here suggests that they are homologues across species. Thus, the bovine protein will also be designated as E-FABP in this report. The degree of identity with murine KLBP (76%) does leave the possibility that it is a homologue to this protein instead or that KLBP and E-FABP are names for the same protein.

As a putative member of the FABP family, bovine E-FABP would be expected to bind fatty acids. This hypothesis was supported by the elution of bovine E-FABP with labeled fatty acid during its isolation from testis on an anion-exchange matrix. Since the ability to bind fatty acids is believed to be central to the physiological function of these proteins, the capacity of E-FABP to bind fatty acids was more carefully examined using the fluorescent probe ADIFAB. A number of common physiological fatty acids were tested for their ability to bind recombinant bovine E-FABP. Typical Scatchard plots are shown in Figure 3, and the final results of the ADIFAB assay are shown in Table 1. The unsaturated fatty acid palmitate (C16:0) had the highest affinity and linolenate (C18:3) the lowest affinity. However, all fatty acids tested had relatively high affinities, ranging from 27 to 97 nM.

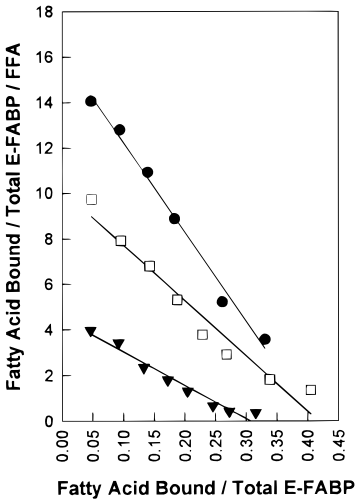

FIGURE 3: Scatchard plot analysis of ADIFAB binding data. Shown are plots representing typical results for palmitate (●), docosahexaenoate (□), and linoleate (▼), where FFA = free fatty acid.

Table 1: Ligand-Binding Analysis of Bovine E-FABP[a]

fatty acid	K_d (nM)
palmitic acid (C16:0)	27 ± 3
oleic acid (C18:1)	36 ± 4
linoleic acid (C18:2)	63 ± 8
linolenic acid (C18:3)	97 ± 12
arachidonic acid (C20:4)	66 ± 8
docosahexaenoic acid (C22:6)	39 ± 5

[a] All experiments were performed at pH 7.4 at 37 °C as described in Experimental Procedures. Values are reported as an average of four or more experiments ± SEM.

The number of binding sites observed was less than 1.0 (data not shown), which has also been the case for heart FABP (H-FABP) (*11*). As with H-FABP, we were not able to identify a single factor which would explain this. Potential factors may include an overestimation of protein concentration, dimerization of the protein that would leave one binding site inaccessible, and partial denaturation of the protein. The cause, though, is most likely a combination of factors.

A previous binding study performed with human E-FABP using non-delipidated protein purified from psoriatic lesions gave results that differed from those reported here (*30*). The apparent K_d obtained for oleate by a saturation experiment was 460 nM, a value that is considerably greater than the 36 nM determined in this study. In addition, the relative affinity for other fatty acids, determined by PAGE/radiobinding and PAGE/autoradioblotting, was reported to be linoleic > oleic > linolenic > palmitic > arachidonic (compare to the values in Table 1). These differences

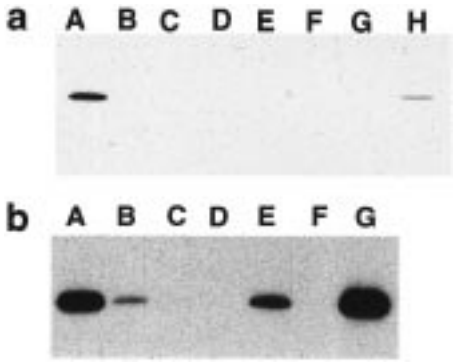

FIGURE 4: Antibody specificity and tissue distribution of E-FABP. (a) Specificity of affinity-purified 1497αFABP IgG. Lanes: A, bovine E-FABP (50 ng); B, rat ALBP (5 μg); C, rat H-FABP (5 μg); D, rat I-FABP (5 μg); E, myelin P2 (5 μg); F, rat TLBP (5 μg); G, mouse KLBP (0.050 μg); H, mouse KLBP (0.500 μg). (b) Tissue distribution of bovine E-FABP. Shown is a scanned image of Western blot analysis using the ECL detection system. Cytosolic proteins were run at 40 μg/lane. Lanes: A, retina; B, testis; C, lung; D, liver; E, heart; F, kidney; G, lens.

probably are due to the different assay conditions and the lack of delipidation of the protein in the previous work.

In addition to understanding its ligand-binding properties, the distribution of bovine E-FABP in tissues and its cellular localization could suggest much about its potential physiological role. To address these issues, polyclonal antibodies were raised against a peptide predicted to encode the putative first β-sheet (based on the conserved tertiary structures that have been solved for other members of the family). This region also was selected in an attempt to create a specific antibody, since it had less conservation of primary structure when compared to other similar family members. The affinity-purified IgGs (1497αFABP) were tested for their ability to recognize other members of the FABP family by Western blot analysis (Figure 4a). With 100-fold greater amounts of protein loaded as compared to the bovine E-FABP standard, no bands were visible for ALBP, H-FABP, intestinal FABP (I-FABP), myelin P2, or TLBP. A faint band was visible for mouse KLBP at a 10-fold excess of protein compared to the standard but was not detected when an equal amount to the standard was loaded. The antibody, therefore, appears to be specific for bovine E-FABP with only minor cross-reaction with the highly similar mouse KLBP. The 1497αFABP IgG was then applied to a multiple tissue Western blot to determine tissue distribution (Figure 4b). Bovine E-FABP was most abundant in lens and retina, moderately abundant in heart and testis, and present in very low amounts in lung. No signal was detected in liver or kidney. The presence of E-FABP in heart and its absence in liver and kidney are consistent with those observed for human E-FABP (*30*).

An estimate of relative abundance was determined by quantitative Western blot. Band intensities of bovine retina, lens, and testis cytosols compared to increasing amounts of recombinant bovine E-FABP were measured by densitometry. (The concentration of pure recombinant E-FABP was determined spectrophotometrically using the calculated extinction coefficient.) Bovine E-FABP was calculated to comprise at least 2.4% of lens, 0.9% of retina, and 0.1% of testis cytosolic proteins. Since E-FABP was restricted to a single cell type in each of these tissues (discussed in the following section), the actual abundance of the protein in those cell types is underestimated by this technique, which measured the abundance in the whole tissue.

Immunohistochemistry was used to determine the cellular localization within the tissues from which bovine E-FABP was isolated. Using 1497αFABP IgG, strong staining was observed in the Müller cells of the retina (Figure 5a). Occasionally, weak staining was also observed in the cytoplasm of large ganglion cells. No staining was observed using flow-through IgG from the E-FABP affinity column (data not shown). For further confirmation and more specific localization of this protein, bovine retina tissue was also examined at the level of electron microscopy, using immunogold labeling. From these studies it was clear that the end feet of the Müller cells were highly labeled; label was also observed in the microvilli. In contrast, the pigment epithelium, photoreceptors, and retinal neurons did not show labeling (Figure 6). The weak staining of large ganglion cell cytoplasm observed by light microscopy could not be confirmed at the electron microscopic level.

Immunohistochemistry of bovine testis showed staining in the Sertoli cells of the seminiferous tubules and no staining in germ cells or Leydig cells (Figure 5b). Flow-through IgG from the E-FABP affinity column did not produce staining (data not shown). This localization was clearly different from that seen for other FABPs in the testis. For example, TLBP is localized to the late germ cells (*8*) and H-FABP to the Leydig cells of the interstitium (*31, 32*).

DISCUSSION

Although many functions have been proposed for members of the FABP family (*1*), their precise physiological role still is not clear. The number of FABP family members, their varying distribution, and their stage-specific expression imply that each serves a unique function. Information regarding ligand-binding characteristics and cellular localization, while not definitive, suggests much about the potential roles of these proteins within the cells in which they are expressed.

There are distinct differences in the affinities different FABPs display for various fatty acids. In this study, we examined the ability of bovine E-FABP to bind a variety of physiological fatty acids. The fluorescent probe ADIFAB, which has been used in studies with a number of other FABPs within the last several years, was employed for this purpose. Since this approach does not depend on binding to fatty acid analogues or on physical separation techniques but rather is performed under conditions of equilibrium with natural fatty acids, the true affinities are probably more accurately reflected. Affinities of various FABPs which have been tested (I-FABP, H-FABP, ALBP, and L-FABP) for a number of saturated and unsaturated fatty acids have now been shown to range from 2 to 480 nM (*11*). These affinities are consistent with a role of FABPs acting as intracellular buffers, since extracellular levels of fatty acids have been estimated to be ∼7 nM (*33*−*36*).

Although bovine E-FABP bound the fatty acid with the lowest solubility and palmitate with the greatest affinity, it was also very effective at binding the more soluble long-chain polyunsaturated fatty acid DHA. Since E-FABP is able to bind both saturated and unsaturated fatty acids with relatively high affinity, E-FABP may be involved in main-

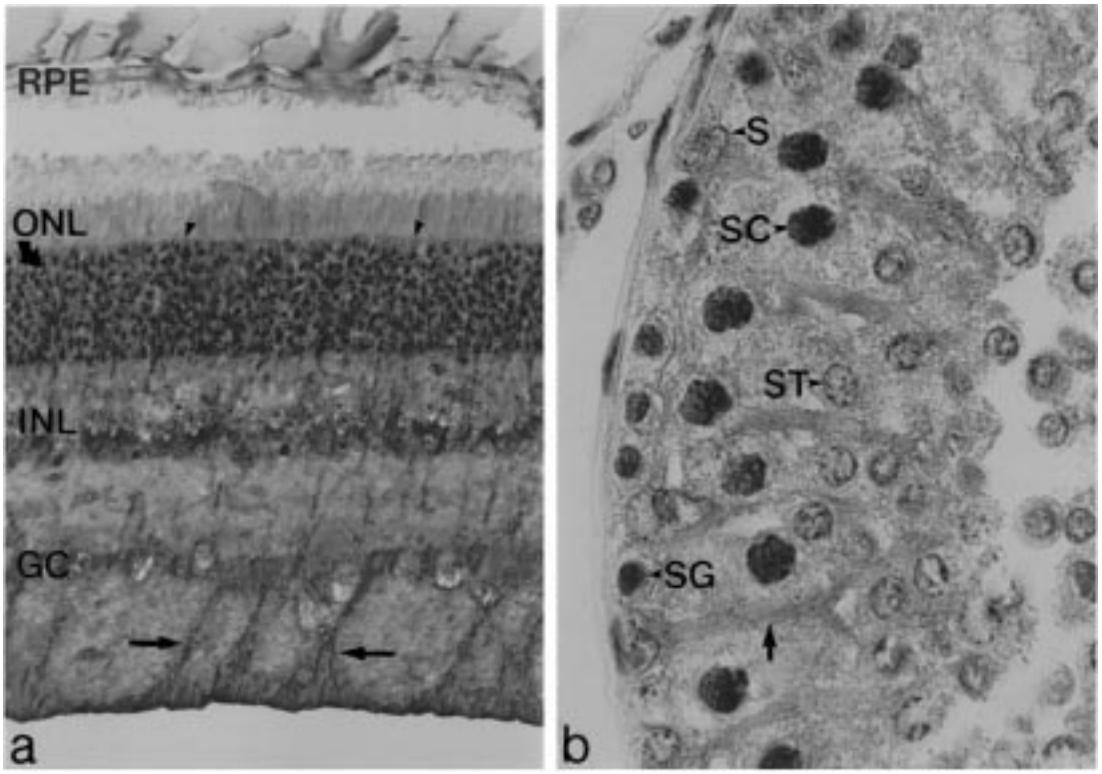

FIGURE 5: Immunolocalization of E-FABP in bovine neurosensory retina and testis. Brown staining indicates the presence of the antigen. Sections were counterstained with hematoxylin. (a) Müller cell staining extending the length of the neurosensory retina from the apical microvilli (arrowheads) to the end feet (arrows). The inner nuclear layer (INL) contains the stained cell bodies of Müller cells. Note the lack of staining in the retinal pigment epithelium (RPE) and in the cell bodies of the photoreceptors in the outer nuclear layer (ONL). Occasional weak staining can be seen in the cytoplasm of large ganglion cells (GC). Magnification 320×. (b) Stained Sertoli cell processes (arrow) extend between cords of germinal epithelium in the seminiferous tubule. The main cell body of the Sertoli cell (S) is also stained. In contrast, the spermatogonia (SG), spermatocytes (SC), and spermatids (ST) are unstained, as are the Leydig cells. Magnification 800×.

taining a supply of several different fatty acids essential for cell nourishment. Although bovine E-FABP was originally isolated because of its ability to bind DHA, the level of affinity for DHA compared to that for other fatty acids and the cellular localization of E-FABP suggest that it is not exclusively a DHA-binding protein. The binding and transport of DHA, however, may still be an important function of E-FABP. Brain lipid-binding protein (BLBP) has been shown to bind DHA with higher affinity than it does for other fatty acids (*37*). It would be interesting to determine whether BLBP is also present in retina and testis, where DHA is also abundant.

In addition to ligand-binding data, the cellular localization of FABPs yields valuable clues as to potential physiological roles. Some comparisons can be made about the cell types in which E-FABP was localized. Both cell types are considered to be supporting cells for the tissues in which they are located. Sertoli cells, via their tight junctions, form a "blood-testis barrier", isolating all but the most immature germ cell type from the general circulation. All nutrients, therefore, must pass through the Sertoli cells to reach the more mature germ cells. Müller cells, as supportive cells, come into contact with every other cell type in the retina, including the capillary endothelial cells, enveloping each with their cytoplasmic processes and supplying them with glucose and other nutrients. While much is known about the ability of Müller cells to regulate ion channel function and neurotransmitter uptake, a great deal is still unknown about the many functions of the Müller cell, including lipid metabolism.

The localization of E-FABP in supportive cells may be considered analogous to the localization of cellular retinol-binding protein (CRBP), which is also a member of the intracellular lipid-binding protein family. CRBP is abundant in the Sertoli cells in the testis (*38, 39*) and is found in the Müller cells as well as the retinal pigment epithelial cells in the retina (*40, 41*). It is thought that CRBP is involved in the transfer of retinol from the blood stream across the cells forming the blood−tissue barriers (*42*). We propose that E-FABP may be involved in a similar way, providing fatty acids required for the growth and function of the cells supported by the Müller and Sertoli cells. In particular, the Müller cell may be uniquely situated, being in contact with both the interphotoreceptor space and the choroidal capillaries in the retina, to provide the essential nutrient DHA to neurons and photoreceptors. Interestingly, the retinal pigment epithelium, which is known to provide many nutrients to photoreceptors, was not positive for E-FABP.

Other evidence indicates that E-FABP may also be important during development and differentiation. As glial cells in the developing retina, Müller cells probably have some role in neuron orientation. In addition, previous studies on bovine LP2 and human E-FABP have shown that expression of this protein is increased as the lens epithelium differentiates (*24*) and in psoriasis, where differentiation is abnormal (*25, 30*). Although it is not known whether bovine E-FABP levels increase in developing retina, it is tempting to speculate that E-FABP may play some role during development and differentiation in the retina as well.

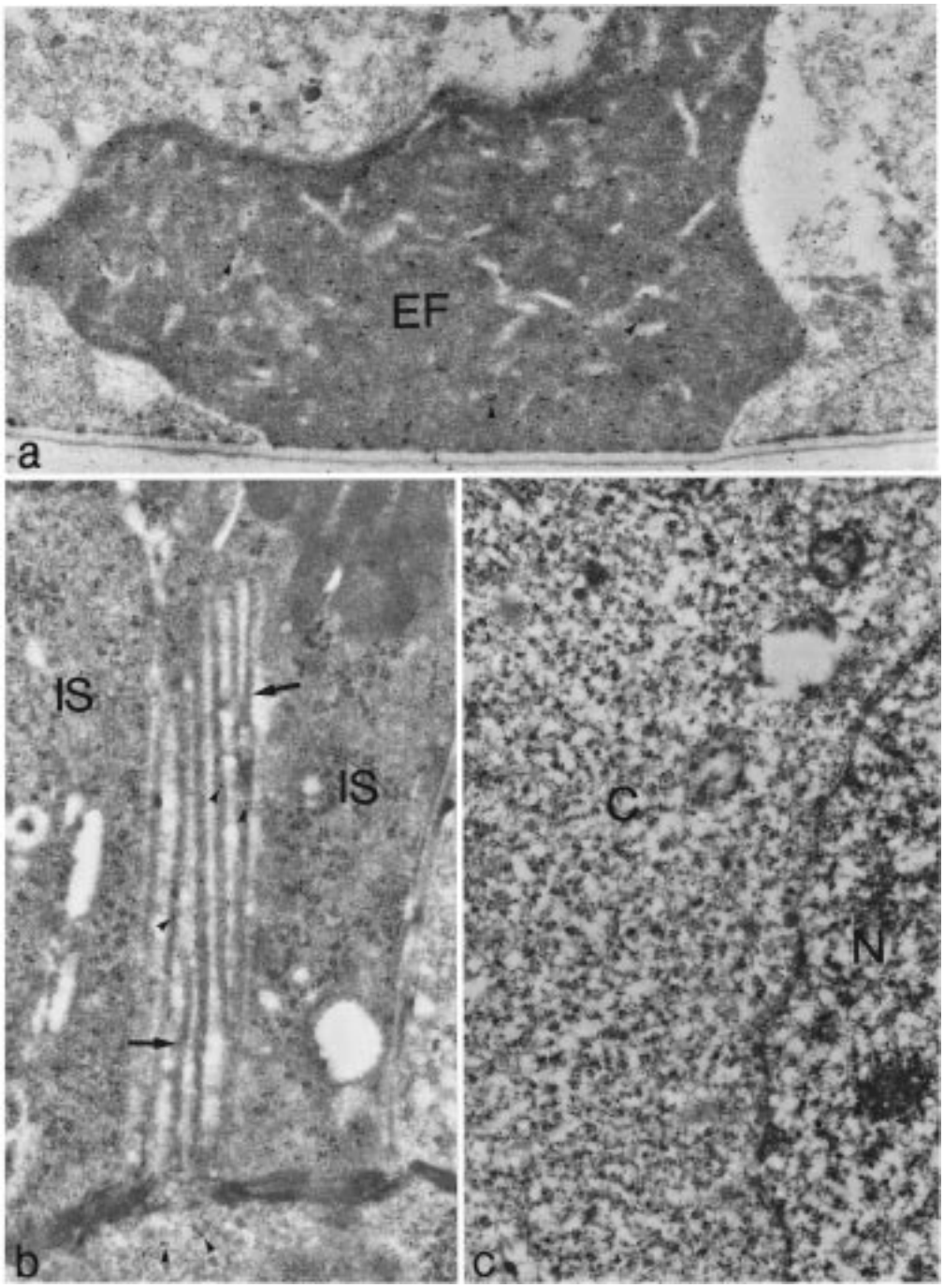

FIGURE 6: Electron microscopic immunocytochemistry of E-FABP in the bovine neurosensory retina. (a) Müller glial cell end foot (EF) ending on the inner (vitreal) surface of the retina. Immunogold particles (arrowheads) indicate the presence of E-FABP. (b) Müller cell apical microvilli (arrows) representing the outermost extent of this cell, which extends across the neurosensory retina. Immunogold particles (arrowheads) are visible in the cytoplasm of these structures and in the cytoplasm of the Müller cell proximal to the so-called outer limiting membrane, a complex of intercellular junctions between photoreceptors and Müller cells. Photoreceptor inner segments (IS) on either side of the Müller cell microvilli are not stained. (c) A portion of the nucleus (N) and cytoplasm (C) of a ganglion cell. Ganglion cell cytoplasm is rich in polyribosomes which should not be confused with immunogold particles. These and all other retinal neurons were unlabeled. Magnification 12925×.

To date, at least nine distinct FABPs have been identified, each with its own spectrum of binding affinities and tissue distribution. Differences in ligand-binding characteristics and modes of interactions with membranes have been examined extensively to distinguish functional differences between the many binding proteins. In addition to providing functional differences, the presence of multiple genes for binding proteins with similar binding properties may allow for expression to be regulated in a cell-specific way and be responsive to different stimuli. For example, liver FABP expression is restricted to liver, intestine, stomach, and kidney (*43*) and has been observed to increase with an increase in dietary fat content (*44*, *45*), after treatment with sex steroid hormones (*46*), and after administration of certain hypolipidemic drugs (*47*). In contrast, the expression levels of other binding proteins are not necessarily affected by the same stimuli. The presence of multiple family members, therefore, allows for a modulation of lipid metabolism to fulfill the needs of given cell types, even those within the same tissue. The elucidation of the cis- and trans-acting factors for

E-FABP would be very informative in furthering our understanding of its physiological role.

In conclusion, we have isolated the bovine homologue of E-FABP and localized it to the Müller cells in the retina and Sertoli cells in the testis. Its localization and its ability to bind fatty acids with varying degrees of saturation all with relatively high affinity suggest that it may supply fatty acids essential for the nourishment of the surrounding cell types.

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