Human Adipocyte Lipid-Binding Protein: Purification of the Protein and Cloning of Its Complementary DNA[†,‡]

Cheryl A. Baxa, Ronald S. Sha, Melissa K. Buelt, Anne J. Smith, Valerie Matarese, Laurie L. Chinander, Kyria L. Boundy, and David A. Bernlohr*

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

Received March 16, 1989; Revised Manuscript Received July 3, 1989

ABSTRACT: Human adipocyte lipid-binding protein (H-ALBP) was purified from normal subcutaneous adipose tissue to greater than 98% homogeneity, utilizing a combination of acid fractionation, gel filtration, covalent chromatography on activated thiol–Sepharose 4B, and anion-exchange chromatography. Human ALBP comprised about 1% of total cytosolic protein in human adipose tissue, had a relative molecular mass of about 15 kDa, and existed as a monomer in solution. The amino terminus of H-ALBP was blocked to sequencing. When a liposome ligand delivery assay was used, H-ALBP saturably bound oleic acid with about 1 mol of ligand bound per mole of protein. Additionally, H-ALBP saturably bound retinoic acid as determined by the quenching of intrinsic tryptophan fluorescence. A full-length H-ALBP cDNA has been cloned; the sequence predicts a 649-base mRNA comprised of a 62-base 5′-noncoding region containing an 18S ribosome-binding site, a single 396-base open-reading frame, and a 191-base 3′-noncoding region. Comparative sequence analysis indicated that the 132 amino acid H-ALBP is a member of a multigene family of intracellular lipid-binding proteins and contains the consensus substrate phosphorylation sequence for tyrosyl kinases.

Adipose tissue possesses perhaps the most active lipid metabolism of any tissue. Adipocytes respond to insulin by the activation of glucose transport, the synthesis of fatty acids, and the esterification of fatty acids into triacylglycerol (Flatt, 1970). Following lipolytic stimulation, adipocytes hydrolyze cellular triacylglycerol depots and efflux fatty acids. The flux of fatty acids within adipocytes is therefore under specific and exquisite hormonal control, though the biochemical mechanisms of fatty acid trafficking from one intracellular locale to another are largely unknown. It is postulated that the intracellular fatty acid binding proteins serve as cytosolic shuttles, solubilizing hydrophobic ligands and delivering them to the appropriate metabolic system for utilization. Included in this multigene protein family are the fatty acid binding proteins (FABPs)[1] from liver (Sweetser et al., 1986; Chan et al., 1985), intestine (Lowe et al., 1987), heart (Said & Schulz, 1984; Offner et al., 1986), kidney (Lam et al., 1988), myelin tissue (Ishaque et al., 1982), mammary gland (Bohmer et al., 1987), and murine adipose tissue (Bernlohr et al., 1984; Hunt et al., 1986).

Insulin promotes lipid synthesis and storage in adipocytes. The mechanism of insulin action requires initial association of insulin with its receptor in the plasma membrane. After insulin binds to the insulin receptor, several biochemical events are induced, albeit on widely different time scales. These include glucose transport and oxidation, and lipid and protein synthesis (Rosen, 1987). However, it is clear that the earliest event to occur following insulin binding to its receptor is autophosphorylation of the receptor. Autophosphorylation of the insulin receptor results in the activation of the receptor tyrosyl kinase activity toward secondary intracellular substrates. Bernier et al. (1987) have shown that within 60 s following insulin addition to phenylarsine oxide (PAO)-treated murine 3T3-L1 adipocytes, a 15-kDa polypeptide (pp15) becomes phosphorylated on a tyrosyl residue. Recently, Hresko et al. (1988) have isolated the 15-kDa polypeptide from PAO-treated 3T3-L1 cells and determined that a tryptic peptide of pp15 is identical in sequence with that of residues 10–20 of M-ALBP. Tyr^{20} of M-ALBP is preceded by several acidic residues, as is seen at the site of phosphorylation in other tyrosine kinase substrates (Hanks et al., 1988; White & Kahn, 1986). The consequences of such a phosphorylation event remain to be elucidated, but the modulation of intracellular lipid trafficking by insulin-dependent phosphorylation of the lipid carrier remains an attractive possibility. This hypothesis would suggest that some structural relationship exists between the phosphorylation site and the ligand-binding domain.

The regulation of human adipose metabolism and steady-state triacylglycerol levels is a complex interaction of hormonal, metabolic, and genetic determinants. It is not known whether human adipose tissue contains an intracellular lipid carrier nor what relationship exists between insulin, insulin resistance, obesity, and the modulation of lipid trafficking. Non-insulin-dependent diabetes mellitus (type II diabetes) is clinically characterized by resistance to the molecular actions of insulin (Olefsky et al., 1982; Reaven, 1988). Insulin resistance, therefore, could involve any of several biochemical defects related either to the insulin receptor or to the proteins with which the insulin receptor interacts. Accordingly, a defect in the ability of the insulin receptor to phosphorylate its intracellular substrates may manifest itself as insulin resistance

[†] This work was supported by National Institutes of Health Grant DK 36979 and by a Presidential Young Investigator Award to D.A.B.

[‡] The nucleic acid sequence in the paper has been submitted to GenBank under Accession Number J02874.

* Author to whom correspondence should be addressed at the Department of Biochemistry, University of Minnesota, 1479 Gortner Ave., St. Paul, MN 55108.

[1] Abbreviations: ALBP, adipocyte lipid-binding protein; H-ALBP, human adipocyte lipid-binding protein; M-ALBP, murine adipocyte lipid-binding protein; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; DEAE, diethylaminoethyl; RA, retinoic acid; PEG, poly(ethylene glycol); pp15, phosphorylated protein 15 kDa; FABP, fatty acid binding protein; PAO, phenylarsine oxide; L-FABP, liver fatty acid binding protein; CRABP, cellular retinoic acid binding protein; I-FABP, intestinal fatty acid binding protein.

0006-2960/89/0428-8683$01.50/0

(White et al., 1988; Maegawa et al., 1988). To address the biochemical basis of insulin action and its relationship to intracellular lipid trafficking, we have examined human adipose tissue to determine if a lipid-binding protein similar to other members of the multigene family is present. The availability of human ALBP would allow for a reconstitution of insulin-dependent phosphorylation in vitro. An analysis of the ligand-binding properties and primary sequence of human adipocyte lipid-binding protein (H-ALBP) may suggest which residues are involved in the ligand-binding domain and the relationship between tyrosine phosphorylation and ligand binding. We describe here our purification of the human adipocyte lipid-binding protein, the characterization of its ligand-binding properties, and the deduction of its primary sequence from the cloned cDNA.

Experimental Procedures

Materials. [9,10-^{3}H(N)]Oleic acid (9 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mol) were obtained from New England Nuclear Corp. Unlabeled oleic acid was purchased from Nu Check Prep, Inc. (Elysian, MN). *all-trans*-Retinoic acid, *N*-acetyl-L-tryptophanamide, poly(ethylene glycol) (approximate MW = 8000), cholesterol, egg yolk phosphatidylcholine, Sephadex G-100, DEAE-Sephadex, and activated thiol–Sepharose 4B were purchased from Sigma. Lipidex 1000 [(hydroxyalkoxypropyl)dextran] was obtained from Packard. Protein standards for electrophoresis were acquired from Pharmacia. Dialysis tubing with 6000–8000 molecular weight cutoff was purchased from Spectrapor. All other supplies were reagent grade.

Purification of H-ALBP. Human subcutaneous fat was obtained following an abdominal lipectomy from a healthy Caucasian female undergoing cosmetic surgery. The tissue was immediately stripped of skin, frozen on dry ice, and stored at −20 °C until use. The frozen tissue (typically 100 g) was thawed, minced into small pieces, and freed of connective tissue. All procedures were performed at 4 °C unless otherwise specified. Any adhering blood was removed by rinsing the minced tissue several times in buffer A (10 mM sodium phosphate, pH 7.0, 20 mM NaCl, 1 mM EDTA, and 0.1 mM PMSF) and filtering through cheesecloth. The washed tissue was homogenized (Polytron, Brinkmann Instruments) in 400 mL of buffer A using five to seven 30-s bursts with 30-s cooling. The opaque homogenate was centrifuged at 3000*g* for 20 min in a Beckman JA-10 rotor, and an extract of soluble proteins was obtained by filtering the supernatant directly through several layers of cheesecloth. The particulate lipid cake was largely retained by the cheesecloth. The bulk of the soluble lipid in the extract was removed by passage of the sample through a 35-mL Lipidex 1000 column at 25 °C equilibrated in buffer A (1 mL/min).

The partially delipidated extract was titrated to pH 5.0 with glacial acetic acid and incubated overnight at 4 °C with gentle stirring. A soluble protein extract was obtained by centrifugation of the sample at 5000*g* in the Beckman JA-10 rotor for 60 min. The supernatant was concentrated by ultrafiltration (Amicon YM5 membrane), clarified by centrifugation, and fractionated by using Sephadex G-100 (2.5 × 40 cm column) equilibrated in buffer B (50 mM NaOAc, pH 5.0). Human ALBP was identified in the column fractions by its characteristic migration in an SDS–polyacrylamide gel and by immunoreactivity with anti-murine ALBP antibodies. The fractions eluting from the Sephadex G-100 column containing human ALBP were pooled and exhaustively delipidated by batch chromatography with Lipidex 1000 resin. Lipidex 1000 was equilibrated in buffer B and added to the G-100 pool (10% w/v). The slurry was incubated for 3–4 h at 37 °C with gentle rotation. The protein was recovered by filtration through silanized glass wool, chilled to 4 °C, and titrated to pH 8.0 using 1 M Tris base. Activated thiol–Sepharose 4B resin, equilibrated in degassed buffer C (10 mM Tris-HCl, pH 8.0, and 100 mM NaCl) at 4 °C, was incubated with the protein solution using a 10-fold excess of thiol-reactive groups to cysteinyl residues. Protein concentration in the sample was estimated spectrophotometrically assuming an $E^{1\%}_{280nm} = 10$. The reaction vessel was flushed with N_2, sealed, and incubated at 4 °C with gentle rotation. After 48–72 h, the slurry was poured into a column at 25 °C, and any nonbinding protein was washed from the resin with buffer C. The bound protein, which included ALBP, was eluted with buffer C containing 25 mM dithiothreitol. The eluted protein was dialyzed once against buffer C containing 1 mM dithiothreitol and several times against buffer C alone. The thiol–Sepharose-eluted protein was applied to a DEAE-Sephadex column (2–4-mL bed volume) equilibrated in buffer C at 25 °C. Contaminating proteins bound to the DEAE-Sephadex resin, and purified human ALBP was identified in the nonbinding fractions by SDS–polyacrylamide gel electrophoresis and immunochemical analysis.

Gel Electrophoresis and Immunochemical Analysis. The progress of the purification was monitored by SDS–polyacrylamide gel electrophoresis as described by Laemmli (1970). The stacking gels contained 3% acrylamide, while the separating gels were 5–15% linear acrylamide gradients. The protein bands were visualized with $AgNO_3$ using the procedure of Morrissey (1981). Immunochemical analysis of SDS–polyacrylamide-resolved proteins was performed according to the procedure previously described (Bernlohr et al., 1985), using a Polyblot transfer system (American Bionetics).

Oleic Acid Binding. The fatty acid binding properties of H-ALBP were determined by using the liposome-binding assay developed by Brecher et al. (1984), as described by Matarese and Bernlohr (1988). Briefly, stock liposomes were prepared containing 40 mM phosphatidylcholine, 13 mM cholesterol, and varying concentrations of [^{3}H]oleate, ranging from 0.15 to 3.0 mM. In the assay, liposomes were diluted 15-fold and incubated with H-ALBP. After a 60-min incubation at 25 °C, the liposomes were pelleted by centrifugation at 400000*g*. Binding was quantitated as the difference in radioactivity in the supernatant of a protein-containing sample relative to a buffer blank. Because of the inability to adequately quantitate the free ligand concentration, the binding results are expressed in terms of a $K_{0.5}$ value. The $K_{0.5}$ value is not formally equivalent to the dissociation constant (K_d); however, it is calculated by expressing the data as $[\text{oleic acid}]_{total} = [\text{oleic acid}]_{bound} + [\text{oleic acid}]_{free}$. Because the hydrophobic ligand exists in several states (i.e., monomeric, liquid-crystalline, micellar, liposome, in the lipid bilayer, and at the air–water interface), the true free ligand concentration is difficult to determine (Small, 1986). $K_{0.5}$ values are therefore useful measures of relative binding affinity within an assay but cannot be compared between assays.

Retinoic Acid Binding. Retinoic acid binding was assayed following the procedure of Cogan et al. (1976) using a Perkin-Elmer 650-10S fluorescence spectrophotometer to measure fluorescence intensities. Fluorometric titrations of H-ALBP with retinoic acid (RA) were performed with the excitation and emission wavelengths set at 285 and 330 nm, respectively. Working solutions of RA were freshly prepared for each experiment by dilution of a 10 mM stock retinoate in absolute ethanol; concentrations were determined spectrophotomet-

rically by using a molar absorption coefficient at 350 nm of 45 000 M^{-1} cm^{-1} (Cogan et al., 1976). The concentration of the protein sample was obtained spectrophotometrically by using the extinction coefficient determined for murine ALBP (Matarese & Bernlohr, 1988). The $K_{0.5}$ value was determined from the corrected binding isotherm assuming 1 mol of RA bound per mole of protein. RA binding to H-ALBP, as assessed by the liposome delivery assay, yielded a stoichiometry of about 1 mol of RA per mole of protein. However, the standard errors were large, which made determination of the $K_{0.5}$ value difficult. For this reason, we adopted the fluorescence assay, which was much more quantitative.

Isolation of Human Adipocyte RNA. Frozen human adipose tissue (1 kg) was thawed in 1000 mL of a buffer containing 4 M guanidine isothiocyanate, 100 mM sodium citrate, 1% sodium sarcosyl, and 5 mM 2-mercaptoethanol, pH 7.0 (buffer S), and the connective tissue was removed. The tissue was minced with sterile scissors and homogenized by using six 30-s bursts of a Polytron tissue homogenizer (Brinkmann Instruments) with 30-s cooling at 4 °C. The homogenized sample was subjected to centrifugation at 7000*g* for 30 min at 4 °C in a Beckman JA-10 rotor. The fat cake was gently removed by aspiration, and the solubilized liquor was filtered twice through 10 layers of cheesecloth to remove any remaining traces of the fat cake. The sample was divided into 100-mL aliquots and transferred into sterile glass 250-mL centrifuge bottles. An equal volume of chloroform/methanol (2:1 v/v) was added to each bottle, and the samples were thoroughly mixed. The suspension was subjected to centrifugation for 30 min at 4 °C on a speed controller setting of 20 in an IEC BE-50 centrifuge. The organic phase was discarded, and the lipid extraction procedure was repeated twice. After organic extraction, the nucleic acids in the aqueous phase were precipitated by the addition of 0.1 volume of 2.0 M sodium acetate and 5 volumes of absolute ethanol. The ethanolic solution was incubated at −20 °C for 12–24 h and the precipitate collected by centrifugation in an IEC centrifuge using the BE-50 rotor as described. The material soluble in ethanol was discarded, and the pellets were immediately resolubilized in 30 mL of buffer S. The RNA extract was subjected to centrifugation at 175000*g* for 30 min at 4 °C in a Beckman 80 Ti rotor to remove debris. A 6-mL aliquot of the supernatant was immediately layered over 5 mL of a solution containing 4.4 M CsCl/7.7 mM EDTA, pH 8.0, and subjected to centrifugation at 190000*g* for 16 h at 20 °C in a Beckman SW-40 swinging-bucket rotor. The pelleted RNA was washed 6 times with ice-cold 80% ethanol and resuspended in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Three hundred micrograms of total RNA was isolated from 1 kg of fresh adipose tissue.

Isolation of Poly(A+) RNA and Library Construction. Polyadenylated RNA was isolated as described by Aviv and Leder (1972), and a human adipocyte cDNA library was prepared by Stratagene, Inc. (La Jolla, CA). Double-stranded cDNA ranging from 500 to 10 000 bp was ligated with *Eco*RI linkers and inserted into the unique *Eco*RI site of λzap. A total of 8×10^6 independent recombinants were prepared.

Northern Analysis of Human RNA. Five micrograms of total RNA from either 3T3-L1 murine adipocytes or human subcutaneous adipose tissue was denatured with glyoxal and separated by agarose gel electrophoresis as previously described (Maniatis et al., 1982). The RNAs were transferred to GeneScreen (Du Pont) and hybridized with ^{32}P random-primer-labeled murine ALBP cDNA (Feinberg & Vogelstein, 1983). Hybridization of the ^{32}P-labeled murine ALBP probe to the human ALBP RNA was conducted for 40 h in 50% formamide, 0.6 M NaCl, 60 mM sodium citrate, 50 mM sodium phosphate, pH 6.8, 0.5 mg/mL $NaPP_i$, and 1% SDS at 37 °C. Following hybridization, the filter was washed twice for 30 min at 42 °C in 0.3 M NaCl, 30 mM sodium citrate, and 1% SDS and twice for 30 min at 50 °C in 150 mM NaCl, 15 mM sodium citrate, and 1% SDS. The hybridized filter was air-dried and subjected to autoradiography at −70 °C using intensifying screens.

Isolation of a Human ALBP cDNA Clone. To identify a human ALBP cDNA, the murine ALBP cDNA (Bernlohr et al., 1984) was radiolabeled with [α-^{32}P]dCTP by the procedure of Feinberg and Vogelstein (1983) and used for hybridization. The hybridization was carried out at 32 °C in 1 M NaCl, 20 mM Pipes, pH 6.5, 50% formamide, 0.5% SDS, and 10 μg/mL sonicated salmon sperm DNA. Following the hybridization, the nitrocellulose filters were washed twice for 45 min with 0.3 M NaCl, 30 mM sodium citrate, and 0.1% SDS at 50 °C, then air-dried, and placed on Kodak X-OMAT AR film for autoradiography at −70 °C. From the approximately 30 000 plaques initially screened, a single hybridizing plaque, termed λHS, was identified. The clone was sequenced by the dideoxy method (Sanger et al., 1977) and was found to be 551 nucleotides in length, lacking the 5′-noncoding region, the initiation codon, and nucleotides coding for about 15 amino-terminal amino acids. To obtain a full-length clone, the cDNA library was rescreened, using the λHS cDNA insert as a hybridization probe. This second screening was performed in a manner identical with that of the first, except that hybridizations were performed at 42 °C, and the filters were washed at 55 °C. From about 20 000 plaques screened with the λHS probe, 6 hybridizing plaques were identified. The recombinant harboring the largest cDNA insert, termed λH-ALBP, was selected for sequence analysis.

Results

Purification of Human ALBP. A purification protocol for ALBP from human adipose tissue was developed based upon an immunochemical assay. We utilized polyclonal antisera raised against murine ALBP to determine if human adipose tissue contained a protein antigenically similar to the murine lipid-binding protein. We were able to detect a similar 15-kDa protein in human adipose extracts, and this allowed us to assess the progress of the purification. A functional assay for monitoring ALBP recovery during various stages of the purification, for example, measuring the binding potential of the extract for [^3H]oleate, was not practical; specific binding could not be measured until after several purification steps due to the large amount of soluble lipid in the crude adipocyte extract. Therefore, the progress of the ALBP purification was routinely assessed by SDS–polyacrylamide gel electrophoresis and by immunochemical analysis. Fractions throughout the purification (see Experimental Procedures) were subjected to electrophoretic separation on a 5–15% SDS–polyacrylamide gel. The separated polypeptides were visualized by silver staining (Figure 1), and ALBP was detected by antibody immunoreactivity. ALBP immunoreactive material copurified with the abundant 15-kDa protein present in the initial extract. When equivalent masses of purified murine and human ALBP were subjected to immunochemical analysis using antisera prepared against the murine protein, human ALBP exhibited approximately 10-fold less antigenicity than did murine ALBP (Figure 2). Equivalent silver staining intensities of the two proteins on an SDS–polyacrylamide gel verified that equal amounts of murine and human ALBP were utilized (data not shown).

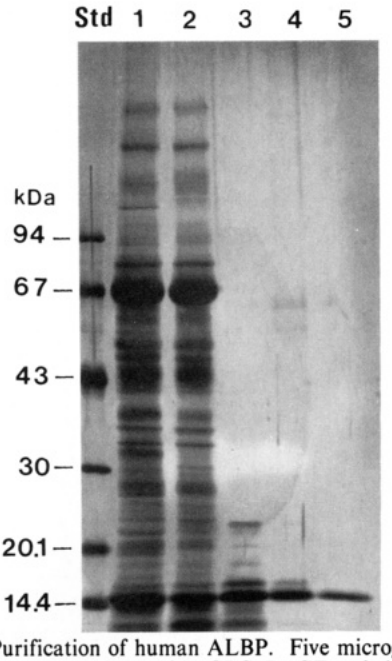

FIGURE 1: Purification of human ALBP. Five micrograms of the indicated protein was separated by SDS–PAGE and visualized with silver nitrate. Std, molecular weight standards; lane 1, human adipose homogenate; lane 2, pH 5 soluble homogenate; lane 3, Sephadex G-100 pool; lane 4, thiol–Sepharose-eluted protein; lane 5, DEAE-purified human ALBP.

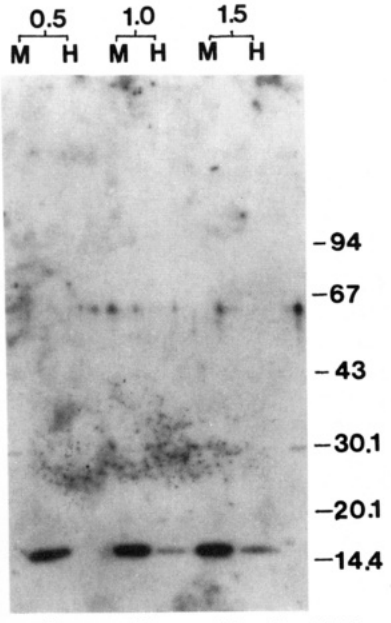

FIGURE 2: Autoradiogram of immunoblot of purified protein. Murine ALBP (M) and human ALBP (H) (0.5, 1.0, and 1.5 μg) were subjected to electrophoretic separation by SDS–PAGE, transferred to nitrocellulose, and subjected to immunochemical analysis using antisera prepared against the murine protein. Detection was accomplished by secondary incubation with ^{125}I protein A.

Ligand Binding. The intracellular lipid-binding proteins bind hydrophobic ligands, either fatty acids or retinoids. To determine the ligand-binding properties of H-ALBP, two assays were utilized: the liposome-binding assay for fatty acids and the fluorescence quenching assay for retinoids. Using the liposome assay of Brecher et al. (1984), human ALBP binds [^{3}H]oleic acid saturably, with a $K_{0.5}$ value of 60 μM. Scatchard analysis indicated that H-ALBP binds approximately 1 mol of fatty acid per mole of ALBP (Figure 3). Previously, a K_d of 3 μM was determined for M-ALBP binding to oleic acid, using the assumption that total ligand concentration equals free ligand concentration (Matarese & Bernlohr, 1988). However, it is not proper to make this assumption, and recalculating our data using the present formalism gave a $K_{0.5}$ value of 60 μM. By calculation of the binding affinity using total ligand concentration minus bound ligand concentration, the $K_{0.5}$ values are reproducible and independent of protein concentration but are greater than those reported by Brecher et al. (1984) or Bass (1985), who assumed total ligand concentration equals free ligand concentration. However, when the ligand in question is relatively insoluble in aqueous solution and can exist in a variety of dispersed forms, the true free ligand concentration is not easily determined. This can lead to a variety of calculated dissociation constants depending upon the amount of protein utilized and the method of assay. This is most clearly seen with hepatic FABP association with oleic acid. Bass (1985) has reported a value of 0.4 μM as the K_d for oleic acid when analyzing 420 nM liver FABP, using the Lipidex assay described by Glatz and Veerkamp (1983). However, from the data presented by Brecher et al. (1984), obtained when the binding of oleic acid to 10 μM L-FABP was assessed by using the liposome delivery assay, an apparent dissociation constant of 15 μM can be calculated. This variability points out the difficulty in assessing the relative binding affinity of a protein for a particular ligand when comparing values determined under different conditions or calculated by different methods.

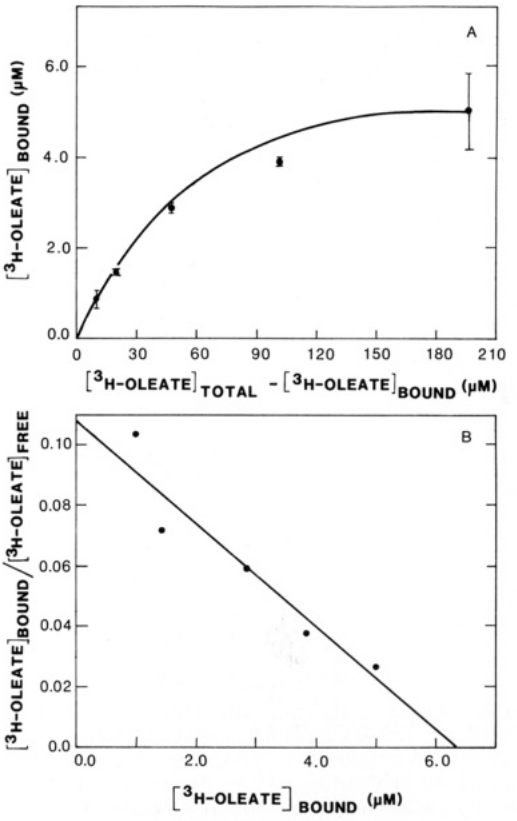

FIGURE 3: Binding of [^{3}H]oleate to human ALBP. Liposomes containing 40 mM phosphatidylcholine, 13 mM cholesterol, and varying concentrations of oleate (0.15–3.0 mM) in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.02% NaN_3 were diluted 15-fold and incubated with 7.6 μM human ALBP for 60 min at 25 °C. Liposomes were pelleted by centrifugation at 400000g at 4 °C, and stoichiometries were determined by the difference in radioactivity in protein-containing samples relative to buffer blanks.

In contrast to the liposome assay used for fatty acid binding, a fluorescence quenching assay was utilized to quantitate retinoic acid binding. The addition of retinoic acid to H-ALBP resulted in a significant (about 50%) decrease in intrinsic

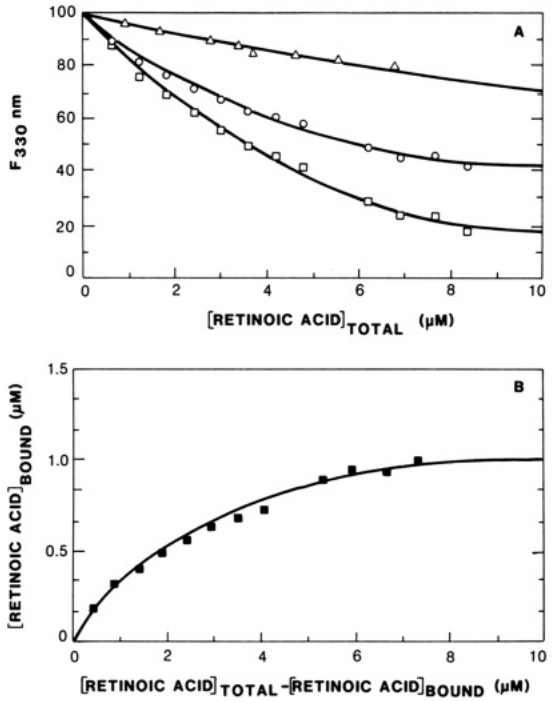

FIGURE 4: Titration of H-ALBP with retinoic acid. H-ALBP (1.14 μM) in 10 mM Tris-HCl, pH 8.0, and 100 mM NaCl was titrated with 0.5–8.5 μM retinoic acid. (A) Corrected fluorescence (○) was determined by subtraction of the fluorescence intensity of *N*-acetyl-L-tryptophanamide (△) from the observed values (□). Excitation wavelength, 285 nm; emission wavelength, 330 nm. (B) The bound ligand was plotted against total retinoic acid concentration minus bound retinoic acid concentration.

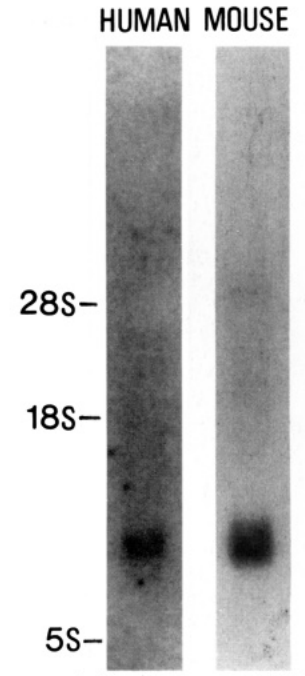

FIGURE 5: Northern analysis of human and 3T3-L1 RNAs. Five micrograms of total RNA from 3T3-L1 murine adipocytes and from human subcutaneous adipose tissue was denatured with glyoxal, separated by agarose gel electrophoresis, and transferred to GeneScreen (Du Pont). The filter was hybridized with 2×10^6 dpm/mL ^{32}P random-primer-labeled murine ALBP cDNA, washed as described, and subjected to autoradiography. The exposure times needed to produce the figure were 6 and 24 h for the murine and human RNAs, respectively.

tryptophan fluorescence (Figure 4A). Assuming $n = 1$, the fractional change in fluorescence can be evaluated as a function of the [retinoic acid]$_{total}$ − [retinoic acid]$_{bound}$ (Figure 4B). Calculation of the $K_{0.5}$ value using this analysis yielded a value of 2 μM. When M-ALBP was fluorometrically titrated with RA, a similar $K_{0.5}$ value was obtained.[2] It is important to note that $K_{0.5}$ values are a function of the method of assay employed and of the mode in which the ligand is delivered to the binding assay. Since it is incorrect to assume that the $K_{0.5}$ value equals the dissociation constant, it is not proper to consider the affinity of H-ALBP for retinoic acid to be greater than its affinity for oleic acid. We were not able to detect any fluorescence quenching or energy transfer upon addition of retinol to H-ALBP. Therefore, H-ALBP specifically binds RA and may contain a tryptophan residue in or near the binding domain. These results indicated that H-ALBP was capable of binding ligands representative of both branches of the multigene family.

Cloning of H-ALBP Complementary DNA. To determine the H-ALBP primary sequence, we chose to clone the H-ALBP cDNA using the M-ALBP cDNA as a hybridization vehicle. Initially, we analyzed total human RNA for the presence of an RNA similar to the murine mRNA to determine whether such a strategy would be feasible. The lipid-binding proteins, as a class, are encoded by mRNAs of about 650–750 nucleotides. Northern analysis of human adipocyte RNA detected an approximately 650–700-nucleotide RNA (Figure 5). The intensity of the hybridization signal was far less than that observed when probing murine adipocyte RNA, suggesting the existence of a related but somewhat different DNA sequence. This finding was consistent with the immunochemical results. Given that an H-ALBP RNA cross-hybridized with the M-ALBP cDNA, albeit weakly, we proceeded with the screening of a human adipocyte cDNA library. One clone, λHS, was isolated in the initial screening; upon sequencing, its cDNA insert was found to contain only 551 nucleotides and was lacking codons corresponding to the amino-terminal 15 amino acids. To obtain a full-length clone, the λHS cDNA was used to screen the library a second time, and six strongly hybridizing plaques were identified. The cDNA insert from each plaque was liberated with *Eco*RI endonuclease and analyzed on a 1% agarose gel. The clone containing the largest insert, termed λH-ALBP, was sequenced (Figure 6). The H-ALBP clone contains a 649 base pair insert comprised of a 62-nucleotide 5′-noncoding region, a 396-nucleotide open-reading frame, and a 191-nucleotide 3′-noncoding region. Comparison of the H-ALBP nucleotide sequence to that of M-ALBP indicated that there is 45% and 86% sequence similarity between the noncoding and coding regions, respectively, of H-ALBP and murine ALBP, explaining the weak hybridization observed on Northern analysis. Computer-aided translation of the open-reading frame indicated that the amino acid sequence of H-ALBP shows 92% sequence similarity with M-ALBP (Figure 6). Conserved between the two proteins are two cysteinyl residues, Cys^2 and Cys^{118}, and two tryptophan residues, Trp^9 and Trp^{98}. Also present in H-ALBP is the tyrosyl kinase phosphorylation sequence surrounding Tyr^{20}, suggesting that H-ALBP may be subjected to insulin-stimulated phosphorylation in a fashion similar to M-ALBP (Bernier et al., 1987; Hresko et al., 1988).

Discussion

In response to insulin, adipocytes synthesize and store triacylglycerol, thereby generating a large lipid depot to be used as an energy reserve during nutritional deprivation. The fatty acid moieties used by adipocytes in the production of tri-

[2] L. Chinander and D. Bernlohr, unpublished results.

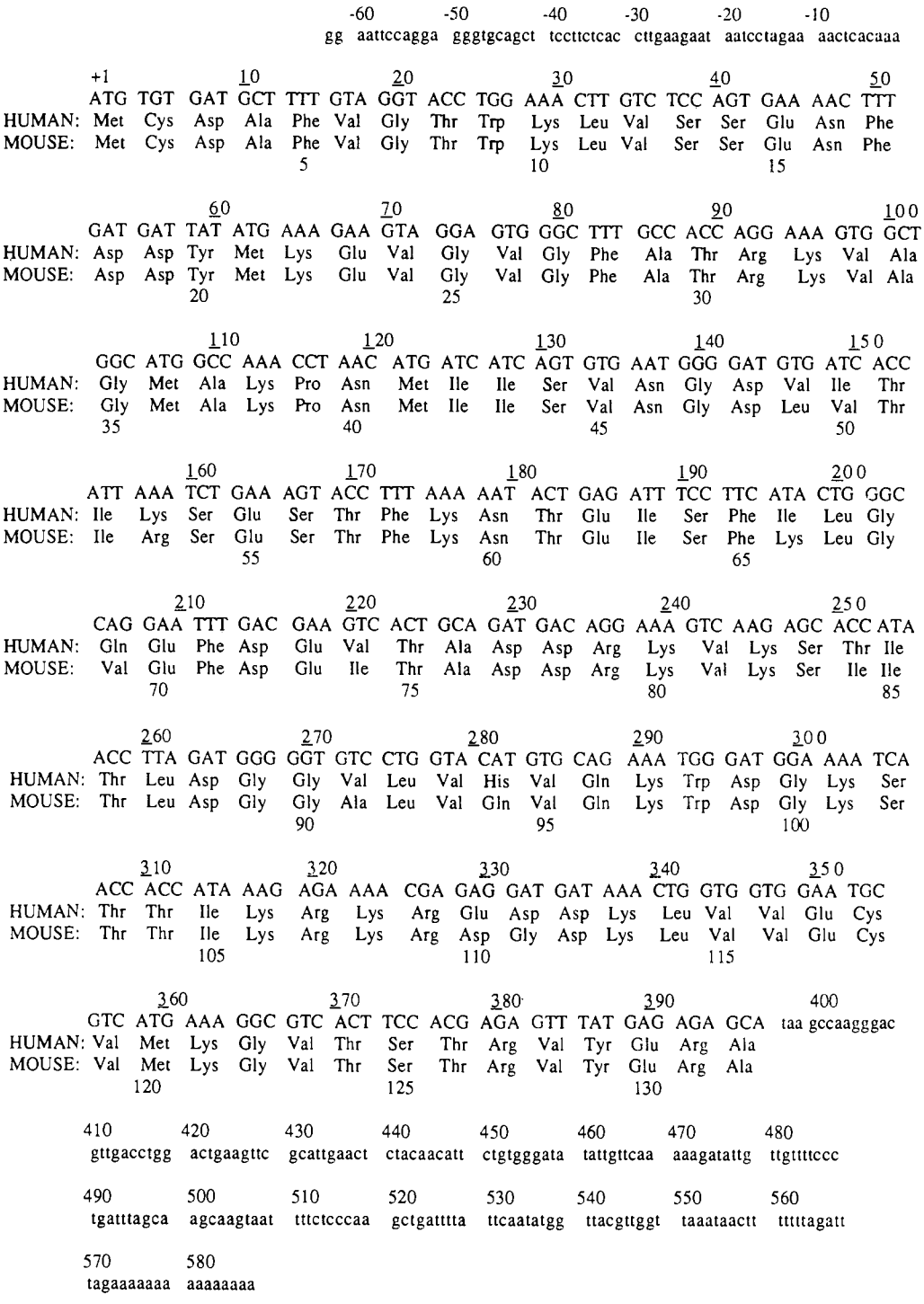

FIGURE 6: Nucleotide and predicted protein sequence of H-ALBP. The amino acid sequence deduced from the 649-nucleotide sequence of human ALBP cDNA (λH-ALBP) was determined via the dideoxy sequence method. The sequence of M-ALBP is shown for comparison.

acylglycerol are derived both from de novo synthesis and from extracellular dietary sources. Adipocytes synthesize and secrete a cell surface lipoprotein lipase that hydrolyzes nutritionally derived triacylglycerol, liberating unesterified fatty acids destined for uptake by the adipocyte. In response to epinephrine or glucagon, adipocytes hydrolyze intracellular triacylglycerol and efflux fatty acids from the cell. Unesterified fatty acids that are liberated are complexed with albumin and traverse to the hepatocyte for oxidation.

The exact mechanism by which fatty acids shuttle between the plasma membrane and the triacylglycerol storage site of the adipocyte remains unclear. The intracellular fatty acid binding proteins are postulated to serve a variety of roles in intracellular lipid metabolism, most notably in the intracellular solubilization and delivery of these unesterified, hydrophobic fatty acids to various locales.

The cultured 3T3-L1 adipogenic cell line has been used as an in situ model system for studying the role of the intracellular lipid-binding proteins in lipid trafficking. Murine ALBP has been purified from 3T3-L1 cells and has been shown to bind both retinoic acid and fatty acids in vitro (Matarese & Bernlohr, 1988). M-ALBP is phosphorylated on Tyr^{20} in situ in response to insulin (Hresko et al., 1988). It is reasonable to hypothesize that phosphorylation may affect either the ligand binding properties of ALBP or its ability to interact with other proteins within the cell. Phosphorylation may therefore provide a mechanism for rapidly affecting the direction of lipid flux. This proposal assumes that lipid-binding

proteins in adipose tissue, as a class, have similar ligand-binding properties and are capable of undergoing tyrosyl phosphorylation. To examine this point, we chose to purify the human ALBP, to determine its ligand-binding characteristics, and to examine its primary sequence. By assessing the conservation of certain amino acids, most notably cysteinyl, tryptophanyl, and tyrosyl residues, we may be able to develop the relationship between the site of phosphorylation and the ligand-binding domain.

The purification of H-ALBP was accomplished by a combination of acid fractionation, gel filtration, covalent chromatography on thiol–Sepharose 4B, and ion-exchange chromatography. H-ALBP was delipidated with Lipidex 1000 resin during the purification. As a consequence of this procedure, H-ALBP purifies as the apoprotein form, devoid of any bound fatty acid. Gas chromatographic analysis of organic solvent extractions of H-ALBP did not reveal the presence of significant quantities of fatty acids. Additionally, the UV–vis spectrum of H-ALBP does not indicate the presence of any bound chromophore, such as a retinoid. H-ALBP, an abundant protein, comprises about 1% of total soluble protein of human adipocytes. It migrates on SDS–polyacrylamide gel electrophoresis with an apparent molecular mass of about 15 kDa (Figure 1), consistent with the deduced molecular mass of 14 701 daltons. The calculated p*I* of H-ALBP is 7.6. Direct protein sequencing of H-ALBP yielded no detectable PTH-amino acid, indicating that the amino terminus was blocked to sequencing. It has been demonstrated that the amino terminus of murine-ALBP is posttranslationally modified, with the terminal methionyl residue removed and the following residue, cysteine, acetylated.[3] Blocked amino termini are commonly found in the lipid-binding protein family, the blocking group often being an acetyl moiety (Sacchettini et al., 1986; Lowe et al., 1987; Dempsey et al., 1981).

H-ALBP saturably bound hydrophobic ligands in vitro. When a liposome delivery assay was used, purified H-ALBP saturably bound 1 mol of fatty acid per mole of protein with a $K_{0.5}$ value of 60 μM. The liposome assay of Brecher et al. (1984) was proven to be simple to manipulate and quantitative over a variety of ligand and protein concentrations. A $K_{0.5}$ of 60 μM for oleic acid binding to H-ALBP was calculated by using a 200-fold range in oleic acid concentration. The value $K_{0.5}$ for oleic acid is significantly greater than the apparent dissociation constant reported for hepatic or intestinal FABP. It is unclear whether this represents true variances in the binding affinities among the proteins of the various cell types or instead differences in the experimental protocols and methodological analysis. Experiments are in progress to assess the relative binding affinities of the various proteins for fatty acids under identical conditions.

The binding of RA to H-ALBP was quantitated via measurement of the reduction of intrinsic tryptophan fluorescence. H-ALBP exhibited saturable binding of retinoic acid when a fluorescence assay was used. The addition of retinol to H-ALBP did not result either in a decrease in intrinsic tryptophan fluorescence or in energy transfer, indicating little, if any, association of this retinoid with H-ALBP. The $K_{0.5}$ value for retinoic acid was determined to be 2 μM, a binding affinity similar to that of M-ALBP using the fluorescence assay.[2] It is not known which tryptophan residue or residues are quenched upon ligand binding.

To determine the primary sequence of H-ALBP, we chose to clone its cDNA. Initially, the 672-nucleotide M-ALBP cDNA was used to probe total human RNA for the presence of similar sequences. A 650–700-nucleotide transcript present in the total human RNA sample was detected; however, a 24-h autoradiographic exposure time was necessary for detection of the H-ALBP mRNA. Given that the murine probe was capable of detecting a human counterpart, we screened a human adipocyte cDNA library. The 649-nucleotide cDNA was identified and verified by direct sequencing. It contains a 62-nucleotide 5′-untranslated region, followed by a start codon and a 396-nucleotide open-reading frame with an in-frame stop codon and then a 191-nucleotide 3′-untranslated region. The 5′-untranslated region contains six consecutive nucleotides between −40 and −35 which perfectly complement the eukaryotic 18S ribosome site near the 3′ end of its RNA (3′...A-C-U-A-G-G-A-A-G-G-C-G-U-C-C-...5′) (Hagenbuchle et al., 1978). The 3′-untranslated region contains the consensus polyadenylation signal sequence, beginning at nucleotide 560 (Proudfoot & Brownlee, 1976).

An examination of the primary amino acid sequence of H-ALBP revealed that it is a member of the intracellular lipid-binding protein family (Sweetser et al., 1987). H-ALBP is most similar to the mammary-derived growth inhibitor (MDGI; 67% in frame identity), while it possesses only 33% sequence identity with intestinal FABP. The crystal structures of rat I-FABP and of bovine myelin P2 protein have been determined (Sacchettini et al., 1988; Jones et al., 1988). In the structures, there is evidence of a noncovalently bound fatty acid within an opening between two β-strands formed by amino acids from the carboxyl domain of the protein. In the I-FABP crystal structure, the indole side chain of Trp^{83} is in close proximity to the fatty acid. The tertiary structures of I-FABP and myelin P2 protein are quite similar to those determined for several other hydrophobic ligand-binding proteins (Newcomer et al., 1984; Holden et al., 1987; Sawyer, 1987) although they have little primary structure similarity. This structural motif, shared by various hydrophobic ligand-binding proteins, has been referred to as a "β-clam".

A goal of this study was to determine whether the human lipid-binding protein shared sequence homology with other lipid-binding proteins in the region surrounding Tyr^{20}, suggesting that it may be a substrate in vivo for the insulin receptor tyrosyl kinase. We have found this region to be very highly conserved; in fact, myelin P2 (Ishaque et al., 1982; Suzuki et al., 1982), mammary-derived growth inhibitor (Bohmer et al., 1987), and heart FABP (Sacchettini et al., 1986) all possess a consensus tyrosyl kinase phosphorylation sequence. Tyr^{20} of H-ALBP lies within the conserved amino-terminal domain at a position in the predicted topological map that may allow for accessibility to the ligand-binding domain. This would predict that tyrosyl phosphorylation may affect fatty acid binding. Interestingly, type II diabetes is associated with elevated free fatty acid levels in the plasma (Reaven, 1988). If ALBP phosphorylation is necessary for fatty acid trafficking, insulin resistance could manifest itself as an inability to carry fatty acids, perhaps resulting in elevated circulating fatty acid levels.

In summary, the purification, characterization, and cloning of human adipocyte lipid-binding protein have been completed. The human protein is a member of the multigene family of lipid-binding proteins. H-ALBP possesses a number of amino acids, most notably cysteinyl and tryptophanyl residues, which are conserved between the human and the murine proteins. The human ALBP contains a consensus tyrosine phosphorylation sequence within the conserved amino-terminal domain. The human insulin proreceptor cDNA has been cloned and

[3] V. Matarese and D. Bernlohr, unpublished results.

expressed in functional form (White et al., 1988; Ebina et al., 1985). Experiments with mutant cloned insulin receptors have shown that the tyrosyl kinase function is necessary to mediate certain of the insulin receptor's functions (Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987). The availability of cloned H-ALBP now allows for a reconstitution of insulin-stimulated ALBP tyrosyl phosphorylation both in vitro and in situ. These results provide a framework for the analysis of the structure of H-ALBP, an examination of its ligand-binding domain, and the relationship between the human insulin receptor and H-ALBP.

ACKNOWLEDGMENTS

We thank Dr. Frank T. Pilney for surgical removal of human abdominal subcutaneous fat. We thank D. Waggoner for his valuable assistance in the immunochemical analyses and for his kind gift of anti-M-ALBP antibodies and R. Stone for his assistance with Northern analyses.

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