Expression, Purification, and Ligand-Binding Analysis of Recombinant Keratinocyte Lipid-Binding Protein (MAL-1), an Intracellular Lipid-Binding Protein Found Overexpressed in Neoplastic Skin Cells†

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ABSTRACT: The keratinocyte lipid-binding protein (KLBP) has been identified on the basis of nucleotide sequence analysis of its cloned cDNA as a new member of the intracellular lipid-binding protein (iLBP) multigene family. To characterize KLBP and determine its ligand-binding properties, its cDNA was subcloned into *Escherichia coli*, and the protein was overexpressed and purified to homogeneity by a combination of acid extraction, gel permeation, and ion-exchange chromatographies. Purified KLBP exhibited high-affinity binding of the fluorescent hydrophobic probe 1-anilinonaphthalene-8-sulfonate (1,8-ANS), displaying an apparent dissociation constant of 390 ± 90 nM ($n = 0.74 \pm 0.2$). Using an assay based upon displacement of the bound fluorophore, KLBP was found to bind long chain fatty acids most avidly; oleic acid (18:1) bound with an apparent K_d of 248 ± 12 nM, and arachidonic acid (20:4) exhibited a dissociation constant of 318 ± 14 nM. As the length of the fatty acid decreased, the binding affinity was reduced; myristic acid (14:0) bound with a K_d of 1409 ± 423 nM, but medium-chain (decanoic acid, 10:0) and short-chain (octanoic acid, 8:0) lipids were not bound at all. The protein did not bind prostaglandin E_2 with any measurable affinity but did associate with eicosanoids such as 5-hydroperoxy-eicosatetraenoic acid (5-HPETE; K_d of 848 ± 211 nM) and 15-HPETE (K_d of 463 ± 243 nM) and to a lesser extent their hydroxy derivatives, 5-HETE and 15-HETE (K_d of 1560 ± 115 nM and greater than 4 μM, respectively). *all-trans*-Retinoic acid was a weak ligand for KLBP, binding with a K_d of 3600 nM, and *all-trans*-retinol did not displace 1,8-ANS. Molecular modeling of the KLBP sequence upon the X-ray crystal structures of several iLBP's suggested that the side chains of one or more cysteine residues may reside within the putative ligand-binding cavity. Consistent with this, sulfhydryl titration of purified KLBP with 5,5′-dithiobis(2-nitrobenzoic acid) at pH 8.0 in the presence and absence of oleic acid revealed that at least one residue was protected from modification by the fatty acid. These results describe the first purification and characterization of the ligand-binding properties of KLBP and indicate that the protein is a fatty acid binding protein with a tertiary structure likely to be similar to other members of the iLBP multigene family.

The intracellular lipid-binding proteins (iLBP's)[1] are a multigene family of low molecular weight cytosolic polypeptides found in a variety of eucaryotic cells (Banaszak et al., 1994). The iLBP's represent a diverse group of over 20 related proteins that display an ability to bind lipophilic molecules such as long-chain fatty acids, retinoids, or lysophospholipids. Although many members of the iLBP multigene family have only been characterized on the basis of their cloned cDNA's, some members have been extensively analyzed by a variety of molecular and biophysical techniques. Crystallographic studies of the iLBP's reveal a conserved structural motif consisting of a β-barrel formed by 10 antiparallel strands connected by short turns and following the n, $n + 1$ folding rule (Banaszak et al., 1994). Within the β-barrel is found a large, central, water-filled cavity. The hydrophobic ligands are bound within the central cavity, sequestered from the external milieu. The primary function of this class of proteins is thought to be the intracellular solubilization and trafficking of lipid molecules to various compartments within the cell (Matarese et al., 1989).

Recently, differential screening of a murine skin squamous cell carcinoma library resulted in the isolation of a cDNA that is predicted to encode a new member of the iLBP family termed keratinocyte lipid-binding protein, KLBP (previously called Mal-1) (Krieg et al., 1993). The predicted sequence of the KLBP exhibits between 50%−60% identity and 60%−70% similarity with the amino acid sequence of several iLBPs, including the adipocyte LBP (Matarese & Bernlohr, 1988), myelin P2 (Narayanan et al., 1988), testis LBP

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[1] Abbreviations: LBP, lipid-binding protein; FABP, fatty acid binding protein; ALBP, adipocyte LBP; KLBP, keratinocyte LBP; myelin P2, myelin lipid-binding protein; iLBP(s), intracellular lipid-binding protein(s); 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid; BSA, bovine serum albumin; ETYA, eicosatetraynoic acid; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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(Schmitt et al., 1994), brain FABP (Kurtz et al., 1994), heart FABP (Claffey et al., 1987), psoriasis-activated FABP (Madsen et al., 1992), cutaneous FABP (Watanabe et al., 1994), and epidermal FABP (Siegenthaler et al., 1994). In addition, KLBP has significant sequence similarity (~35% identity, 40%−45% similarity) with cellular retinoic acid binding proteins I and II (Krieg et al., 1993). Analysis of the KLBP primary sequence reveals the conservation of several residues that have been shown both crystallographically and biochemically to be necessary for the binding of retinoic acid or long-chain fatty acids. These residues include the triad of R109, R129, and Y131, which creates an electrostatic network with the carboxylate of the bound ligand (Sha et al., 1993; Xu et al., 1993). Surprisingly not present in KLBP is a phenylalanine at position 60, a residue which in the corresponding position of the intestinal and adipocyte proteins affects the dynamics of lipid exchange within the cavity.[2] Given these similarities, it is reasonable to hypothesize that KLBP should bind a lipophilic molecule with an acidic head group. However, the differences in sequence between KLBP and other family members may suggest that the protein has a much more restrictive ligand-binding selectivity.

The tissue distribution of KLBP demonstrates high levels of expression in neoplastic murine epidermis with low levels of expression in normal skin, adipose tissue, and mammary and tongue epithelium (Krieg et al., 1993). All of these tissues are responsive to both fatty acids and retinoids but perform vastly different physiologic roles. Because of this unique tissue distribution we were interested in determining if KLBP could bind retinoic acid and/or fatty acids. In this study we describe the purification of KLBP from overexpressing *E. coli* and characterize the protein as a fatty acid, but not retinoic acid, binding protein. Biochemical characterization of KLBP suggests it possesses a ligand-binding cavity analogous to that for other members of the iLBP family.

EXPERIMENTAL PROCEDURES

Materials. DNA restriction and modification enzymes were purchased from Promega. Double-stranded DNA sequencing was performed using a sequencing kit manufactured by Bethesda Research Laboratories Life Technologies and carried out in a DNA Thermal Cycler model 480 from Perkin-Elmer Cetus. Probes used for Northern analysis were labeled with a Random Primed Label Kit from Boehringer Mannheim. Oligonucleotides were purchased from Integrated DNA Technologies. 1-Anilinonaphthalene-8-sulfonate (1,8-ANS) was purchased from Molecular Probes Inc. Fatty acids were purchased from either Nu Check Prep Inc. or Biomol Inc. Nalidixic acid and all other chemicals used were reagent-grade Sigma products. Molecular cloning procedures were performed using standard methods (Sambrook et al., 1989).

Expression of KLBP cDNA in E. coli. To liberate the entire KLBP cDNA from the plasmid p11CL6 (Krieg et al., 1993) with the restriction endonucleases *Nco*I and *Eco*RI, an internal *Nco*I site was inactivated. Site-directed mutagenesis using the oligonucleotide 5′GCC TTG GCC ATT GCA GCC ATC3′ was used to introduce the mutation c145a into the KLBP cDNA, thereby destroying the internal *Nco*I site. Single-stranded DNA (200 ng) was mixed with 6.7 pmol of phosphorylated c145a oligonucleotide and was annealed during a 45 min incubation period as the sample cooled from 80 to 30 °C. Heteroduplexes were then subjected to *in vitro* DNA synthesis using Klenow fragment. The resultant recombinants were transformed into wild type *E. coli* strain JM 109 to generated pMKB1. Correctly mutagenized plasmids were identified by restriction analysis and verified by direct DNA sequencing. In order to obtain large quantities of recombinant KLBP protein, the cDNA insert from pMKB1 was liberated with *Nco*I and *Eco*RI and subcloned into pJMB100A (Monsanto Corp.) to generate pMKB2, the clone used for all expression studies described within.

Large-Scale Induction and Purification. In order to express KLBP, *E. coli* strain JM 101 harboring pMKB2 was grown in rich medium containing 100 μg of ampicillin/mL. At an optical density of 2 at 600 nm, expression of KLBP was induced by the addition of nalidixic acid (final concentration, 100 μg/mL) for 5 h. Following induction, the cells were recovered by low-speed centrifugation at 8000 RPM for 20 min at 4 °C in a JA-10 rotor. The cells were resolubilized in 50 mL of lysis buffer consisting of 25 mM imidazole (pH 7.0), 50 mM NaCl, 5 mM EDTA (pH 8.0), 1 mM β-mercaptoethanol, 0.1 mM PMSF, 2 μg of pepstatin/mL, 2 μg of aprotinin/mL, and 2 μg of leupeptin/mL and placed on ice. Cells were lysed via sonication using a Biosonik sonicator (Bronwill Scientific). Cell debris was removed from the total extract by centrifugation at 12 000 RPM for 30 min at 4 °C in a JA-17 rotor. The supernatant was titrated with a stock 5% (wt/v) protamine sulfate solution made in lysis buffer to a final concentration of 1% over a 20 min period. The mixture was stirred for another 20 min at 4 °C, and the precipitate was removed by centrifugation at 8000 RPM for 20 min at 4 °C. The supernatant was acidified to a final pH of 5.0 using 2 M NaOAc, pH 5.0, and was stirred overnight at 4 °C. The precipitated material was removed by centrifugation at 8000 RPM for 20 min at 20 °C and concentrated using an Amicon ultrafiltration device fitted with a YM-3 membrane. Approximately 12 mL of concentrated extract was applied to a Sephadex G-75 column (5 × 100 cm) and developed with 12.5 mM HEPES (pH 7.5) and 250 mM NaCl. KLBP containing fractions were identified by SDS−PAGE followed by Western blotting, pooled, and concentrated. The low molecular weight fractions were dialyzed into 50 mM sodium formate (pH 4.2), concentrated, and fractionated with a Pharmacia FPLC system utilizing a Mono S HR10/10 cation-exchange column. KLBP was eluted with a salt and pH gradient consisting of 1.0 M NaCl in 50 mM NaOAc (pH 5.2). Pure fractions of KLBP were identified via sodium dodecyl sulfate polyacrylamide gel electrophoresis, dialyzed into 50 mM sodium phosphate (pH 7.4), and concentrated with Centricon 10 filtration columns (Amicon). Because the absorbance and fluorescence spectral characteristics of KLBP were very similar to those of ALBP and because the protein has the same number of tryptophan and tyrosine residues as does ALBP, the concentration of KLBP was estimated using an extinction coefficient measured for ALBP ($\epsilon_{278} = 1.55 \times 10^4$ cm^{-1} M^{-1}), which is similar to that for other lipid-binding proteins.

[2] C. D. Kane, J. Ory, L. J. Banaszak, and D. A. Bernlohr, manuscript in preparation.

Ligand-Binding Studies. Ligand-binding to KLBP was assessed using a fluorescence-based assay system utilizing the hydrophobic probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS). 1,8-ANS binds within the ligand-binding cavity of the hydrophobic ligand-binding proteins and can be displaced with fatty acids (Kane & Bernlohr, 1996). The probe was dissolved in absolute ethanol, and its concentration was determined spectrophotometrically ($\epsilon_{372} = 8000$ cm^{-1} M^{-1}). Proteins were dialyzed into 50 mM sodium phosphate (pH 7.4) and added in aliquots to ~500 nM 1,8-ANS in the same buffer (final ethanol 0.05%, v/v). The samples were mixed for 1 min under dim light, and the fluorescence was measured in a thermostated (37 °C) Perkin Elmer 650-10S fluorescence spectrophotomer. Relative fluorescence was plotted versus increasing protein concentration, and Scatchard analysis was used to calculate the binding parameters. All values reported were calculated from two to five independent binding isotherms (mean ± standard deviation).

Competition Assays. Various lipids were assessed for their ability to displace 1,8-ANS bound to KLBP. KLBP (0.66 μM) in 50 mM sodium phosphate (pH 7.4) was mixed with 500 nM 1,8-ANS at 37 °C, and the fluorescence signal was determined. Increasing concentrations of competitor lipid (each diluted from a 25 mM stock in absolute ethanol) were added to the KLBP/1,8-ANS complex and mixed for 30 s, and the fluorescence signal was measured. Relative fluorescence as a function of increasing concentration of competitor lipid was determined and analyzed as described by Epps et al. (1995). The midpoint of the assay was defined as the point at which 50% of the initial fluorescence had been lost. The I_{50} was then used to calculate an apparent K_i using $K_i = [I_{50}]/(1 + [L]/K_d)$, where K_i = apparent inhibitor constant (equivalent to the K_d), [L] = free concentration of 1,8-ANS, and K_d = apparent dissociation constant of KLBP for 1,8-ANS.

Sulfhydryl Titrations. Purified KLBP (typically about 5 μM) was dialyzed into 50 mM Tris-HCl buffer at pH 8.0 and incubated with a 20-fold molar excess of DTNB. The production of the thionitrobenzoate anion was monitored continuously using a Beckman DU-70 spectophotometer and quantitated using the extinction coefficient of 13 600 cm^{-1} M^{-1} at 412 nm. For experiments involving fatty acids, lipid was added from an ethanolic stock (final ethanol 0.05%) to a 10-fold molar excess over KLBP.

RESULTS

To characterize the binding properties of KLBP, site-directed mutagenesis was utilized to create a KLBP cDNA that could easily be subcloned into bacterial expression vectors. The full-length KLBP cDNA clone found in the original cloning vector, p11CL6, contained 5′ *Nco*I and 3′ *Eco*RI restriction sites that bracketed the entire coding region. However, before these restriction sites could be utilized, an internal *Nco*I site needed to be inactivated. Site-directed mutagenesis was used to introduce the c145a substitution into the KLBP cDNA, thereby inactivating the internal *Nco*I site while not altering the coding sequence. The *Nco*I−*Eco*RI fragment was subcloned into pJMB100A to produce an *E. coli* expression vector (pMKB2) that placed the KLBP cDNA under control of the inducible recA promoter. This construct yielded expressed protein that could be purified with good yield.

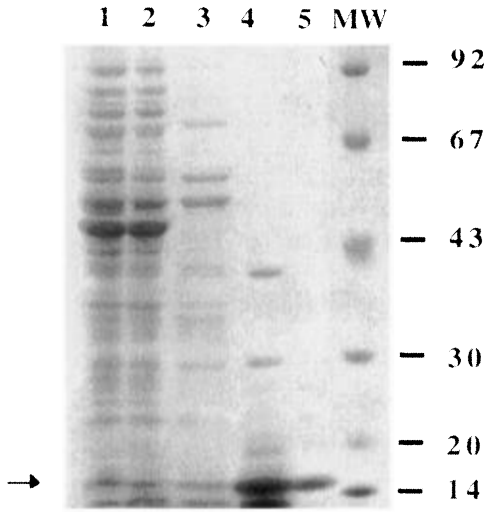

FIGURE 1: Purification of KLBP from bacterial lysates. Protein extracts from *E. coli* were separated by 5%−15% SDS−PAGE and stained with Coomassie Blue. The molecular masses of the standards (in kDa) are found to the right of the figure. Lane 1, total *E. coli* lysate; lane 2, protamine sulfate supernatant; lane 3, pH 5.0 supernatant; lane 4, G-75 fractionated pool; lane 5, FPLC-purified KLBP. The arrow denotes the location of KLBP.

To obtain homogeneous recombinant KLBP, the cells were harvested and the protein was purified using a combination of acid fractionation, gel filtration, and ion-exchange chromatography. As with several other iLBP's, KLBP was soluble in dilute acid (pH 5.0), which allowed for a simple purification step leading to gel filtration. The progress of purification is shown in Figure 1. Protein purity was greater than 95% on the basis of sodium dodecyl sulfate polyacrylamide gel electrophoresis, and typical yields of protein were 5 mg/L of bacterial culture. The protein was soluble at a wide range of pH values and ionic strengths, and its ligand-binding activity was stable for several months when stored at −70 °C. Purified KLBP behaved as a monomer in solution, eluting from the gel filtration column at a position consistent with a low molecular weight protein. In addition, chemical cross-linking studies using bissulfosuccinimidyl suberate (BS^3) indicated that KLBP was most likely monomeric for little if any dimeric KLBP could be detected (results not shown). The intrinsic tryptophan fluorescence spectrum of KLBP was measured as an assessment of its folding. KLBP has two tryptophan residues at positions W11 and W100 that are conserved among many of the lipid-binding proteins and, as such, would be expected to exhibit similar intrinsic tryptophan fluorescence characteristics. The fluorescence excitation and emission maxima were 285 and 334 nm, respectively. These values are very similar to those obtained for other members of the iLBP multigene family, indicating that KLBP was likely to be folded properly.

In order to characterize the ligand-binding properties of KLBP, we analyzed the interaction between the protein and the fluorescent ligand 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS). 1,8-ANS has been used as a general probe for hydrophobicity (Slavik, 1982) and has recently been found to bind members of the iLBP multigene family within the ligand-binding cavity (Kane & Bernlohr, 1996). By extension of those studies, we evaluated the 1,8-ANS binding properties by KLBP. KLBP displayed high affinity for 1,8-ANS, and a representative binding isotherm and corresponding Scatchard plot are shown in Figure 2. Analysis of the

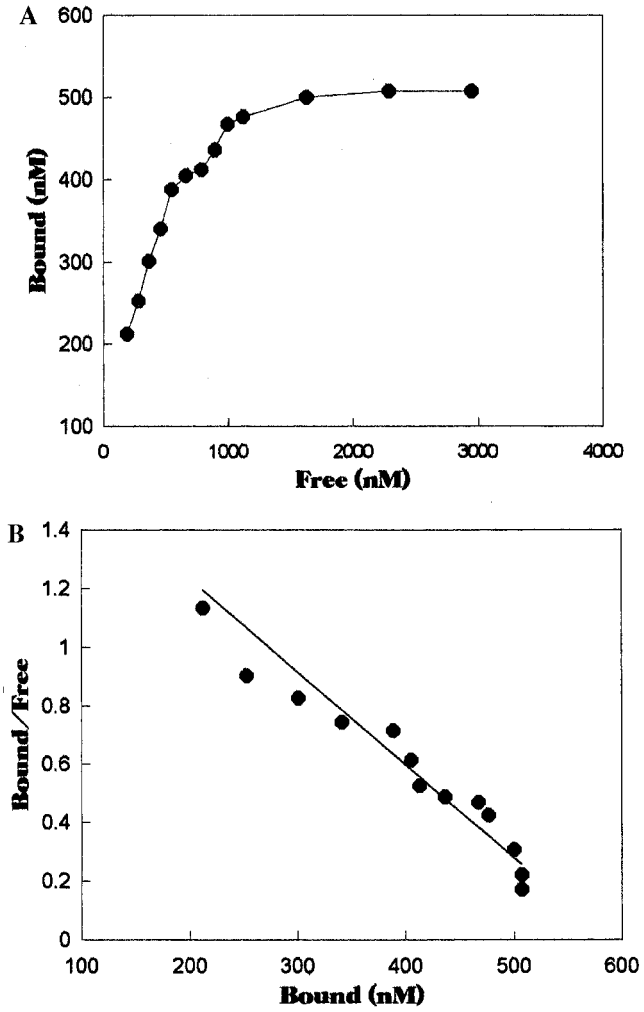

FIGURE 2: 1,8-ANS binding isotherms. Panel A: increasing concentrations of KLBP in 50 mM sodium phosphate (pH 7.4) were incubated with 500 nM 1,8-ANS, and the increase in fluorescence was recorded. Data shown are taken from a single experiment and are representative of three independent experiments. Panel B: data taken from panel A were reanalyzed in a Scatchard plot and yield an apparent dissociation constant of 390 ± 90 and a stoichiometry of 0.74 ± 0.2.

binding isotherms revealed that the protein exhibited an apparent dissociation constant (K_d) of 390 ± 90 nM for 1,8-ANS with an average stoichiometry of 0.74 ± 0.2 mol of 1,8-ANS bound per mol of KLBP.

A competition assay which utilized fatty acids or retinoids to displace 1,8-ANS bound to KLBP was used to assess both ligand-binding specificity and the affinity. The results of the competition studies are shown in Figure 3 as well as the apparent dissociation constants (K_d) that were obtained from those experiments in Table 1. In general, the long-chain fatty acids proved to be strong competitors of 1,8-ANS fluorescence. The ligand with the highest affinity was oleic acid with a K_d of 248 ± 12 nM. However, other 18-carbon fatty acids, linolenic and linoleic acid, were also bound with high affinity, Table 1. Medium-chain fatty acids such as myristic acid bound with only moderate affinity (1409 ± 423 nM), and the short-chain fatty acids decanoic acid and octanoic acid did not displace the bound 1,8-ANS. The very long chain fatty acid, nervonic acid (24:1), also was a poor ligand for KLBP, indicating that only fatty acids in 14−20 carbon range are most effectively bound. The affinity and specificity of KLBP for the retinoids *all-trans*-retinoic acid and *all-trans*-retinol was also assessed. *all-trans*-Retinoic acid was a weak competitor of 1,8-ANS fluorescence, exhibiting a K_d of 3600 nM. *all-trans*-Retinol had virtually no affinity for KLBP, as shown by its inability to displace 1,8-ANS. These findings establish KLBP as a fatty acid binding protein and, to a much lesser extent, as a retinoic acid binding protein. Acyl-CoA's and acyl glycerols were not tested for their binding to KLBP.

Because arachidonic acid derivatives such as prostaglandins and eicosanoids have been implicated in the physiology of neoplastic skin cells and in the development of transformed keratinocytes, we evaluated the binding potential of KLBP for these molecules. Prostaglandin E_2 did not displace 1,8-ANS from KLBP and thus is not a ligand for the protein. In contrast, KLBP associated with high affinity with a variety of hydroperoxyeicosatetraenoic acids (HPETE's) and their hydroxyeicosatetraenoic acid (HETE's) derivatives. As shown in Table 1, the 5- and 15-hydroperoxy derivatives bound with higher affinity to KLBP than did their hydroxy counterparts. Though there was only a 2-fold difference in binding affinity between 5-HPETE and 5-HETE, there was a 10-fold difference in affinity between 15-HPETE and 15-HETE.

Several members of the LBP multigene family have had their crystal structures solved to high resolution (Banaszak et al., 1994). A comparison of such X-ray crystal structures reveals a remarkably similar β-barrel fold for the proteins with their α-carbon tracings virtually superimposable. On the basis of this, we modeled the primary sequence of KLBP upon the X-ray crystal structure backbone of ALBP and noted that two cysteine residues of KLBP were predicted to be within the putative ligand-binding cavity: these were C120 and C127, Figure 4. The primary sequence of KLBP predicts six cysteine residues: two cysteines, C43 and C47, have their side chains oriented away from the ligand-binding cavity while two others, C67 and C87 have their side chains buried in the core of the polypeptide. X-ray crystallographic studies of several lipid-binding proteins in their holo conformation have revealed that the bound fatty acid is in close proximity with the side chains of cysteine residues. Consistent with this, chemical modification of the cysteinyl residues of the adipocyte lipid-binding protein (cysteine 117; Buelt & Bernlohr, 1990) demonstrated that fatty acids could block sulfhydryl modification and that sulfhydryl modification could block fatty acid binding. To determine if KLBP has a cysteine residue(s) at or near the ligand-binding site, sulfhydryl titration of the protein in the presence and absence of fatty acids was conducted. As shown in Figure 5, six cysteine residues could be modified with DTNB in the presence of sodium dodecyl sulfate, consistent with the amino acid sequence derived from the cDNA. In the absence of denaturation, three to four sulfhydryl groups were accesible to modification, implying that some SH cysteinyl residues are inaccesible or unreactive to DTNB modification. When either oleic or arachidonic acids were incubated with KLBP and then the cysteines modified, only two SH groups were reactive. If sodium dodecyl sulfate was included with the fatty acid, all six accessible groups could be revealed. These results suggest that oleic and arachidonic acids protect at least one cysteine residue from modification and support the concept of KLBP having a teritary structure with a binding cavity similar to that for other iLBP's.

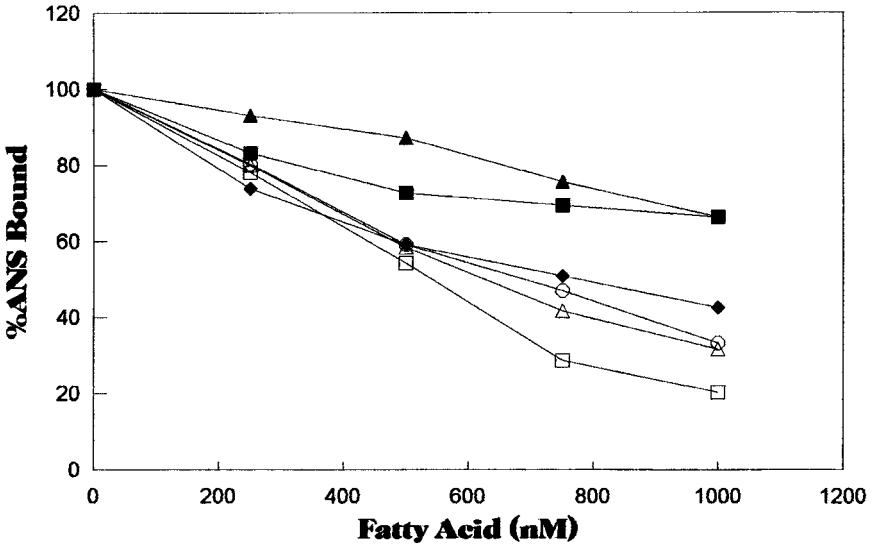

FIGURE 3: Ligand displacement of 1,8-ANS bound to KLBP. KLBP (0.66 μM) was mixed with 500 nM 1,8-ANS in 50 mM phosphate buffer (pH 7.4), and the fluorescent signal was determined. Various concentrations of the indicated fatty acids (delivered in ethanol) were added in aliquots to the protein−lipid complex and mixed for 60 s, and the fluorescence signal was determined: myristic acid (■), palmitic acid (▲), arachidonic acid (○), oleic acid (□), linoleic acid (△), and linolenic acid (◆). Results are shown from a single displacement assay and are representative of at least three trials for each fatty acid.

Table 1: Ligand-Binding Analysis of KLBP[a]

ligand	apparent dissociation constant (nM)
octanoic acid (C8:0)	>10 000
decanoic acid (C10:0)	>8000
myristic acid (C14:0)	1409 ± 423
palmitic acid (C16:0)	802 ± 164
oleic acid (C18:0)	248 ± 12
linoleic acid (C18:2)	313 ± 4
linolenic acid (C18:3)	337 ± 29
arachidonic acid (C20:4)	318 ± 14
nervonic acid (C24:1)	>8000
eicosatetraynoic acid (20:4)	>4000
5(*S*)-hydroperoxyeicosatetraenoic acid	848 ± 211
5(*S*)-hydroxyeicosatetraenoic acid	1560 ± 115
15(*S*)-hydroperoxyeicosatetraenoic acid	463 ± 242
15(*S*)-hydroxyeicosatetraenoic acid	>4000
all-trans-retinoic acid	3600
all-trans-retinol	>10 000

[a] All values reported were determined at pH 7.4 at 37 °C as described in Experimental Procedures.

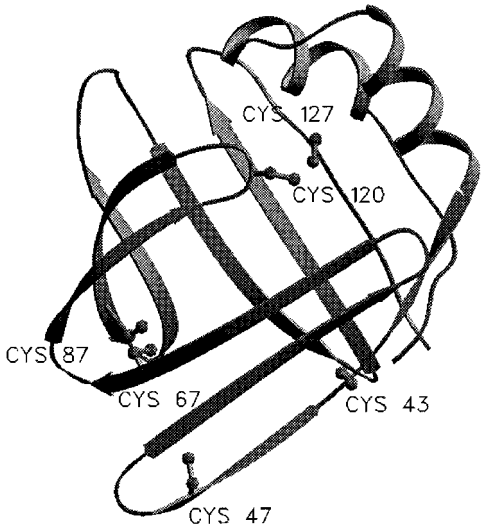

FIGURE 4: Hypothetical model for KLBP showing the β-barrel fold. The primary sequence for KLBP was modeled to that for the adipocyte lipid-binding protein and is displayed as a ribbon diagram. The positions of the six cysteinyl residues are indicated by the stick model. Cys 120 and 127 are predicted to reside within the ligand-binding cavity.

DISCUSSION

The KLBP cDNA was originally cloned and identified by differential screening of a murine squamous cell carcinoma cDNA library. Sequence analysis of this clone indicated that it exhibited significant sequence similarities on both the nucleotide and amino acid level with a family of proteins known as the intracellular lipid-binding proteins. Furthermore, analysis of the predicted KLBP protein sequence with that of several members of the iLBP family revealed that several of the amino acid residues that have been shown to be intimately involved in the binding of long chain fatty acids and retinoic acid are conserved in KLBP. These residues include R109, R129, and Y131, which are needed for electrostatic interactions with the ligand carboxylate. Crystallographic analysis of several members of the iLBP family with bound ligands has shown that many of the residues whose side chains are within 4.5 Å of the bound ligand (F19, M23, A36, K61, A78, D79, R81, I107, V118, and C120) (LaLonde et al., 1994a,b) are all conserved in KLBP. Taken together, this information allowed us to hypothesize that KLBP should function as an acidic lipid, possibly fatty acid or retinoic acid binding protein. However, KLBP does not have a phenylalanine residue at or near position 60. A phenylalanine residue at the corresponding positions of the muscle FABP and adipocyte LBP has been suggested to function as a molecular gatekeeper, regulating fatty acid entry and exit out of the binding cavity. The lack of Phe60 suggests that ligands other than fatty acids may be bound by KLBP. Therefore, the KLBP cDNA was expressed in *E. coli*, and the recombinant protein was purified to homogeneity in order to evaluate its ligand-binding potential.

In order to characterize the ligand-binding properties of KLBP, we used a 1,8-ANS discplacement assay recently developed in our laboratory for the analysis of binding properties by members of the iLBP multigene family (Kane & Bernlohr, 1996). 1,8-ANS had been used as a general

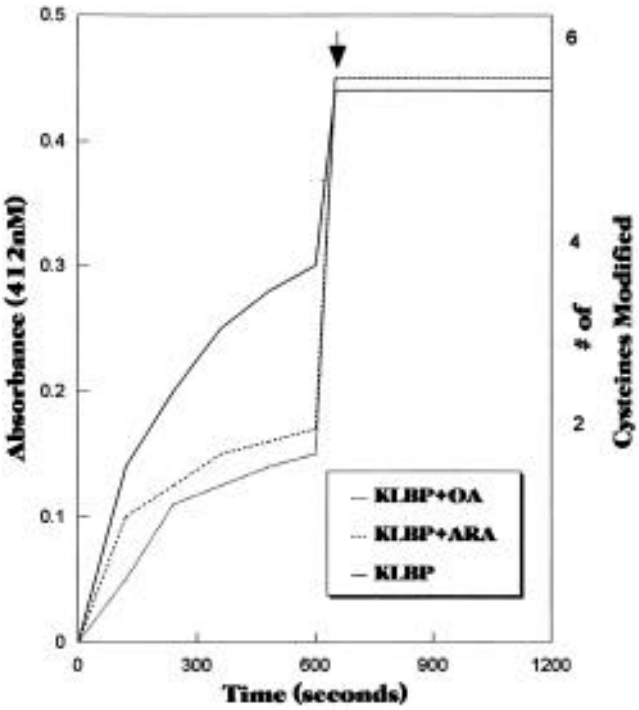

FIGURE 5: Sulfhydryl titration of KLBP in the presence and absence of bound fatty acid. KLBP (5 μM in 50 mM Tris-HCl pH8.0) was incubated in the presence or absence of a 10-fold molar excess of the indicated fatty acid for 10 min at room temperature to reach equilibrium. To initiate the reaction, 5,5′-dithiobis(2-nitrobenzoic acid) was added (100 μM), and the production of the thionitrobenzoate anion was followed spectrophotometrically. The arrow indicates the addition of 1% SDS to the reaction: OA, oleic acid; ARA, arachidonic acid.

purpose probe of hydrophobicity in both lipid membranes and proteins (Slavik, 1982). We exploited this observation and found that 1,8-ANS binds with high affinity within the lipid-binding cavity of ALBP and other members of the iLBP family. Fatty acids bind within the ALBP cavity and block chemical modification of C117, a residue whose side chain is in close proximity to the bound lipid (Buelt & Bernlohr, 1990). Similarly, 1,8-ANS blocks C117 modification by 5,5′-dithiobis-2-nitrobenzoate, a chemical reagent previously shown to specifically modify the cavity cysteinyl residue of ALBP. In addition, preliminary X-ray crystallographic analysis indicates that 1,8-ANS resides within the binding cavity. Lastly, and most obviously, long-chain fatty acids but not short-chain fatty acids or retinoids displace 1,8-ANS from the protein. Therefore, 1,8-ANS was used to assess the ligand-binding ability of KLBP. The 1,8-ANS binding isotherm is shown in Figure 2 and illustrates KLBP's ability to bind the probe with high affinity. KLBP bound 1,8-ANS tightly and displayed an apparent dissociation constant of 390 $\pm$ 90 nM. These results mirrored the binding activity of ALBP for 1,8-ANS, which exhibits an apparent dissociation constant of 400 nM.

Qualitative information concerning the environment of 1,8-ANS bound to KLBP was obtained by examining the excitation and emission maxima of the probe fluorescence. The excitation and emission maxima of 1,8-ANS bound to KLBP were 376 and 474 nm, respectively. The spectral data for KLBP displayed a red shift of 7 and 9 nm for the excitation and emission maxima (respectively) relative to those measured for ALBP and in general for other members of the iLBP family. The existence of a red shift suggests that the polarity of the environment in which 1,8-ANS is bound in KLBP is greater than that bound to other iLBP's. This most likely is related to a greater number of charged and polar residues within the binding cavity as evidenced by the amino acid sequence of KLBP; however, alternate explanations are possible. In general, the ligand-binding cavities of the various iLBP's have large and variable numbers of polar residues. Frequently, amino acid residues with charged and/or polar side chains constitute 50% of the composition of the cavity. Consequently, binding cavities with different polarities is not unexpected.

The ligand-binding characteristics of KLBP were characterized by use of a competition assay which measured the ability of various lipids to displace 1,8-ANS. The competition assays, which are shown in Figure 3 and Table 1, describe both the specificity and affinity of KLBP for a variety of hydrophobic ligands. Long-chain fatty acids such as such oleic acid (C18:1) and arachidonic acid (C20:4) are bound more tightly than retinoic acid, while octanoic acid and retinol are not bound at all. The degree of saturation of the fatty acid ligand appears to have negligible effects upon the measured K_d values. Crystallographic analysis of various members of the iLBP family has revealed that these proteins are characterized by the conservation of a common tertiary motif of a 10-stranded β-barrel. The iLBP's display a large, water-filled central cavity in which the lipid is bound. The significant degree of KLBP sequence similarity with other iLBP's coupled with similar spectroscopic characteristics suggest that KLBP should also share in the β-barrel folding motif. Shared tertiary structure and the conservation of the residues involved in lipid−protein interactions, such as R109, R129, and Y131, would explain why KLBP binds fatty acids such as oleate and arachidonate.

Recently, the cDNA cloning of iLBP's from rat skin (Watanabe et al., 1994) and rat lens epithelium (Wen et al., 1995) were reported. They exhibit 99% amino acid identity to each other and 91% identity to KLBP and therefore may represent the rat homologue of murine KLBP. Two additional iLBP's have been identified in human epidermal tissue, psoriasis-associated FABP (Madsen et al., 1992) and epidermal FABP (Siegenthaler et al., 1994). On the basis of partial amino acid sequencing of epidermal FABP, it has been suggested that these proteins may be identical. Epidermal FABP has been isolated from normal and psoriatic human skin and has been shown to bind stearic acid and oleic acid with high affinity but does not bind arachidonic acid or retinoic acid. The binding properties of epidermal FABP are therefore markedly different from the binding properties of KLBP, which binds arachidonic acid with high affinity and retinoic acid with reduced affinity. Furthermore, the deduced amino acid sequence of psoriasis-activated FABP (Madsen et al., 1992) exhibits only about 80% identity to the amino acid sequence of KLBP and is therefore unlikely to represent the human homologue of KLBP. The different binding characteristics of murine KLBP and human epidermal FABP suggest that they represent two different lipid-binding proteins.

Recent work with the transcription factors known as the peroxisomal proliferator-activated receptors (PPARs) has shown them to be involved in the expression of genes related to adipocyte differentiation and metabolism. Activation of reporter plasmids containing PPAR ligand-binding domains occurs *in situ* when fibroblasts are treated with peroxisomal

proliferators such as ETYA or long-chain polyunsaturated fatty acids (Tontonoz et al., 1994a,b). Like that for ALBP, the KLBP gene has been shown to be transcriptionally activated by fatty acids such as 2-bromopalmitate in Ob1771 cells (Ibrahimi et al., 1994). Furthermore, ETYA has been shown to strongly activate differentiation of fibroblastic cell lines to adipocytes when these cells overexpress PPAR γ2 (Tontonoz et al., 1994a,b). Although only KLBP is expressed in preadipocytes, in mature fat cells ALBP and/or KLBP could serve as the cytoplasmic receptor for peroxisome proliferators like ETYA. However, when ETYA was evaluated for its ability to displace 1,8-ANS from the binding domain of KLBP, no displacement was evident. In contrast, ALBP readily bound ETYA with an apparent binding affinity of 363 $\pm$ 53 nM. Therefore, if iLBP's are cytoplasmic receptors for ETYA or ETYA derivatives, it is more likely that in adipocytes ALBP serves that role rather than KLBP.

The study of lipid metabolism and lipid transport in epidermal cells is important for many reasons. In epidermal cells lipids play an important part by contributing to the skin lipid barrier which regulates transepidermal water loss. Fatty acids, especially arachidonic acid and their oxidized metabolites, such as the prostaglandins and the hydroxyeicosatetraenoic acids (HETEs), were shown to be involved in defense mechanisms, including inflammatory and immune processes, and are thought to be involved in cell signaling processes regulating proliferation and differentiation. Furthermore, dysregulation of the arachidonic acid metabolism in skin has been shown to correlate with pathophysiological processes such as psoriasis and skin cancer. Skin of psoriatic patients contains increased total lipids and phospholipids as well as arachidonic acid and their oxidized products such as 12-HETE (Hussain et al., 1994). The overexpression of psoriasis-activated FABP and epidermal FABP in psoriatic skin supports the concept of an alteration of lipid metabolism in this disease. Dramatically elevated levels of fatty acids, especially products of the lipoxygenase-catalyzed arachidonic acid pathway, such as 12- and 8-HETE, are found in benign and malignant mouse skin tumors (Lehmann et al., 1992; Krieg et al., 1995). Furthermore, an involvement of eicosanoids in neoplastic skin diseases is documented by the inhibitory activities of drugs impairing eicosanoid biosynthesis or preventing the activities of the latter (Furstenberger & Marks, 1990). Also coincidental with the elevated lipoxygenase metabolism, KLBP has been found to be highly overexpressed in mouse skin tumors (Krieg et al., 1993). Although KLBP did not bind 15-HETE to any extent, it did associate with 5- and 15-HPETE as well as 5-HETE, suggesting that the protein has selectivity toward certain arachidonic acid derivatives. The binding of arachidonic acid to KLBP may mediate the intracellular trafficking of arachidonic acid and their lipoxygenase-catalyzed products. Overexpression of KLBP may be mechanistically linked to the increased levels of nonesterified arachidonic acid metabolites in diseased skin.

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