Purification and characterization of the human epidermal fatty acid-binding protein: localization during epidermal cell differentiation *in vivo* and *in vitro*

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Epidermal fatty acid-binding protein (E-FABP) was isolated from human skin and purified to homogeneity. Its molecular mass was estimated to be 15 kDa and the pI of non-denaturing protein was 5.6. Scatchard-plot analysis revealed one class of binding site for oleic acid with a K_d of 0.46 μM. Structure–binding relation experiments revealed a high affinity of E-FABP for stearic acid which decreased on reduction of the number of carbon atoms or introduction of double bonds into the fatty acid chain. Squalene, cholesterol and retinoic acid isomers showed no affinity, suggesting that E-FABP displays high specificity for fatty acids. E-FABP is a scarce cytosolic protein (0.065% of total protein). Only trace amounts could be detected in normal human skin but up to 42.5±3.4 pmol/mg of protein was found in a non-malignant defect of keratinocyte differentiation (psoriatic lesions). E-FABP levels were low in cultured human keratinocytes grown under proliferation-stimulating conditions but increased about 2-fold on induction of differentiation by Ca^{2+}. Immunohistochemical localization showed cytosolic staining in differentiated cells of normal and psoriatic skin, suggesting a link between E-FABP and keratinocyte differentiation. The presence of E-FABP in tissues other than skin (heart, intestine and adipose tissue) excludes its specific role in fatty acid metabolism in epithelial cells or its involvement in skin lipid-barrier function.

INTRODUCTION

The cytosolic fatty acid-binding proteins (FABPs) are widely distributed low-molecular-mass (14–15 kDa) proteins belonging to a protein superfamily that also includes the cytosolic retinoid-binding proteins [1]. Little is known about the precise function of FABPs, but it is generally assumed that they are involved in fatty acid transport and metabolism [1–4]. Thus far, the amino acid sequence of FABPs of heart [5,6] and myelin [7] and the nucleotide sequence of the cDNA of FABPs of the liver [8,9], intestine [10] and adipose tissue [11] have been published. Each FABP displays a characteristic pattern of tissue expression. They are abundantly expressed in tissues that are either subjected to large fluxes of fatty acids or those that have high demands for fatty acids as energy substrates [1]. The study of fatty acid transport in epidermal cells is important for at least two reasons: (i) fatty acids are present in significant amounts in the extracelluar space of the stratum corneum and contribute to barrier function in regulating transepidermal water loss [12], and (ii) *in vivo* keratinocytes lack Δ^5 and Δ^6 desaturases and therefore must obtain essential fatty acids from the circulation [13]. Furthermore, essential fatty acids are involved in the control of cell growth and are important for maintaining membrane lipid structures (see review in ref. [12]).

We have recently reported that keratinocytes contain small amounts of an epidermal FABP (E-FABP) which specifically and reversibly binds fatty acids [14]. E-FABPs display a different electrophoretic mobility from FABPs of other human tissues, such as liver, heart, intestine and adipose tissue, and from cellular retinoic acid- and retinol-binding proteins (CRABP-I, -II and CRBP-I, -II respectively), suggesting a new uncharacterized type of human FABP. The molecular cloning of a gene corresponding to a low-molecular-mass protein from lesional psoriatic skin (PA-FABP) and showing a high similarity (approx. 55%) to myelin FABP has recently been reported [15]; however, no biochemical or binding studies have been performed on this protein. During epidermal differentiation, marked changes in lipid composition take place which are accompanied by progressive deletion of phospholipids and glycosphingolipids, with enrichment in ceramides, cholesterol and free fatty acids [12]. To study their role during cell differentiation, the amounts of E-FABP present at various stages of keratinocyte maturation were measured using the PAGE/radiobinding assay [16,17]: in complete differentiating epidermis (normal human skin), in epidermis displaying incomplete differentiation (psoriasis) and in two distinct populations in cultured human keratinocytes that were undifferentiated and differentiating.

As the highest amounts of E-FABP were found in psoriatic skin, this tissue was used for isolation, purification and characterization and to raise antibodies to E-FABP for immunohistological localization. E-FABP expression was significantly dependent on keratinocyte differentiation both *in vitro* and *in vivo*.

EXPERIMENTAL

Materials

all-*trans*-[11,12-^{3}H]Retinoic acid (50 Ci/mmol), [9,10-^{3}H]oleic acid (10 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (1.67 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]12-hydroxyeicosatetraenoic acid (12-HETE) (215.3 Ci/mmol), [^{3}H]lanosterol (28.5 Ci/mmol), [4,8,12,13,17,21-^{3}H]squalene (30 Ci/mmol), [^{3}H]palmitic acid (60 Ci/mmol), [^{14}C]stearic acid (58 mCi/mmol), [1-^{14}C]linoleic acid (53.0 mCi/mmol), [1-^{14}C]linolenic acid (52 mCi/mmol) and [26-^{14}C]cholesterol (53.8 mCi/mmol) were purchased from Du Pont–NEN. 9-*cis*-[^{3}H]Retinoic acid was a

Abbreviations used: FABP, fatty acid-binding protein; E-FABP, epidermal FABP; PA-FABP, psoriasis-associated FABP; CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; 12-HETE, 12-hydroxyeicosatetraenoic acid.

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generous gift from Dr. A. A. Levin (Hoffmann–La Roche, Nutley, NJ, U.S.A.). The purity of the labelled oleic acid was checked by h.p.t.l.c. analysis and consistently exceeded 98%. All operations involving the retinoids and unsaturated fatty acids were performed under yellow light. Fatty acids and retinoids were dissolved in ethanol containing butylated hydroxytoluene (500 μg/ml) as antioxidant. Fatty acids and retinoids were obtained from Sigma. The other chemicals used were the best commercially available.

Human keratinocyte culture

Keratinocytes isolated from human foreskin were cultured in the presence of irradiated 3T3 fibroblasts using the Rheinwald–Green feeder technique [18] with 5% fetal calf serum. The cells were grown in normal Ca^{2+} (1.2 mM) until the culture stratified forming multilayers. The more differentiated cells were then stripped off by exposure of the culture to low-Ca^{2+} medium (0.06 mM), leaving the undifferentiated cells attached to the dish [19]. Both cell populations were harvested, washed three times with ice-cold isotonic PBS and stored at −70 °C. Cytosolic fractions were obtained as described for tissue preparation (see below). The ability of cells to form cross-linked envelopes and to simultaneously express CRABP-II, enzymes involved in retinoic acid formation from retinol and retinoic acid nuclear receptors was determined and used as a marker of cell differentiation [20]. Detached cells, a high percentage of which spontaneously formed cornified envelopes [21] and which contained the greatest amounts of components involved in retinoic acid metabolism, were termed 'differentiating' cells, whereas attached cells that displayed no or only weak expression of these markers were termed 'undifferentiated' cells [17,19–21].

Extraction of tissue proteins

Psoriatic scales were collected by gently scraping the lesions with a plastic scraper and were stored at −20 °C until used. About 1.5 g of lyophilized scales were homogenized in 10 ml of Tris buffer (50 mM Tris/HCl, 25 mM NaCl, 2.5 mM EDTA, 1 mM dithiothreitol, pH 7.5) using a Polytron tissue homogenizer (Kinematica, Lucerne, Switzerland) and subsequently centrifuged at 100000 *g* for 60 min at 4 °C. The supernatant was used immediately or stored at −20 °C. Protein extraction from other human tissues was performed in the same manner, but without prior lyophilization. The sample protein concentrations were estimated as described in [22] using BSA as standard.

Radiobinding assays

The following standard incubation conditions, used previously for CRABP-I and -II studies [16,17], were used to assay the different ligands on the binding proteins. The alcoholic solutions of labelled ligands were deposited in glass microtubes and evaporated under a stream of N_2. Subsequently, 100 μl of supernatant (containing about 300 μg of protein) was added and the mixture was incubated for 2 h at 22 °C or for 16 h at 4 °C. The actual concentration of ligands used for binding studies is stated in the Figure legends. Competition studies were performed with a 500-fold excess of unlabelled ligand. Samples were then subjected to either PAGE/radiobinding [16] or PAGE/autoradiobinding assays [16,17] to measure the ligand-binding proteins. Analytical experiments were performed two or three times. No substantial differences were observed between individual experiments.

Analytical measurement of binding capacity for E-FABP

PAGE/radiobinding

The supernatants incubated with labelled ligands were subjected to vertical slab PAGE (7.5% acrylamide) in non-denaturing conditions as described previously [16]. By this technique, proteins are separated by their net electric charges and not by their molecular masses! After electrophoresis (when the marker reached the bottom of the gel), the gel was divided into lanes, cut into 2 mm bands and the radioactivity quantified by liquid-scintillation counting.

PAGE/autoradioblotting [16,17]

After electrophoresis, the separated proteins were transferred to a nitrocellulose sheet in 30 mM sodium phosphate buffer, pH 6.5, at 13 °C for 2.5 h under the electrical conditions recommended by the manufacturer (Bio-Rad). The blotted proteins were visualized by direct autoradiography with Hyperfilm-^{3}H (Amersham International, Amersham, Bucks, U.K.). The film was developed after 12 days of exposure.

Purification procedure

Protein supernatants (100000 *g*; 143 mg in 11 ml of buffer) were separated by gel filtration on a Sephadex G-100 column (4.5 cm × 100 cm) equilibrated with 5 mM sodium phosphate buffer containing 0.2 M NaCl, 2 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride and 0.02% sodium azide at pH 7.5. The column was eluted at a flow rate of 0.8 ml/min and fractions of 10 ml were collected. The elution profile of proteins was monitored by a u.v.-absorbance detector set at 280 nm. The elution positions of radiolabelled ligand-binding proteins were assessed in a liquid-scintillation counter by counting the radioactivity in 100 μl portions of each eluted fraction diluted in 4 ml of Ultima Gold (Packard). The column was calibrated using Blue Dextran, albumin, ovalbumin, chymotrypsin, myoglobin, human epidermal CRABP–[^{3}H]retinoic acid and heart FABP–[^{3}H]oleic acid complexes. Fractions containing the radioactive peak eluted at a molecular mass of 15 kDa (elution volume 850–1050 ml) were pooled and concentrated to 19.2 mg of protein in 2.75 ml with an Amicon ultrafiltration device used with 10YM5 membranes (molecular-mass cut-off 5000 Da). Of this, 9.7 mg of protein in 1.4 ml was subjected to a semipreparative PAGE technique under similar conditions to those used for the analytical PAGE/radiobinding technique but with slight modification. A 3 mm spacer was used for gel casting (instead of 1.5 mm) and the sample was applied without a comb. The electric current for one gel (11.5 cm × 14 cm × 0.3 cm) was twice as high as that used for the analytical approach (40 mA, at constant power). Electrophoresis was stopped when the Bromophenol Blue, used as a marker, migrated to 1 cm from the bottom. The band of gel corresponding to E-FABP (R_F = 0.34) was sliced and crushed in a mortar placed in liquid nitrogen. E-FABP was extracted three times from the gel powder with 1.5 vol. of a buffer containing 100 mM Tris/HCl, 0.5 M KCl and 0.05% Triton X-100 at pH 7.5. To separate the gel from the protein solution, the mixture was centrifuged at 5000 *g*. The recovery of protein trapped in the gel was 87 ± 3%. The protein solution was then dialysed against 20 mM Tris/HCl/1 mM dithiothreitol, pH 8.0, with a Spectra/Por dialysis membrane (Spectrum, Houston, TX, U.S.A.), with a molecular-mass cut-off of 3500 Da, and subsequently concentrated to 1 ml in an Amicon concentration cell.

The third step of FABP purification was achieved by injecting 200 μl portions (1.68 mg of protein) into an ion-exchange column, Mono Q HR5/5 (Pharmacia-LKB, Uppsala, Sweden) equili-

brated with 20 mM Tris/HCl at pH 8.0 (buffer A). The column was connected to an h.p.l.c. apparatus (Varian 9010) and the protein was eluted with a linear gradient, with increasing percentages of buffer A containing 0.5 M NaCl at 1 ml/min. The eluted proteins were detected by monitoring u.v. absorption at 280 nm; E-FABP was eluted at 8.8 min. The E-FABP-containing fraction was collected and the protein concentrated as above. Total recovery was about 0.065%, representing a 1660-fold purification.

Preparation of proteolytic peptides

E-FABP was reduced with dithiothreitol and alkylated with 4-vinylpyridine in a 100 μl volume containing 6 M guanidinium chloride, 0.25 M Tris/Cl, pH 8.5, and 1 mM EDTA. After desalting against 0.1% trifluoroacetic acid on a Fast Desalting column, using the Smart System (Pharmacia-LKB Biotechnology, Uppsala, Sweden), the sample was dried in a Speed Vac. The protein was then dissolved in 50 μl of 0.1 M Tris/HCl, pH 9.0, containing 8 M urea and the solution was immediately diluted with 50 μl of 0.1 M Tris/HCl, pH 9.0. LysC-specific protease from *Achromobacter lyticus* was then added to achieve a substrate/enzyme ratio of 100:1. After incubation for about 15 h at 30 °C, the digestion was stopped by acidifying with trifluoroacetic acid. Generated peptides were isolated by reversed-phase chromatography using the Smart System equipped with a μRPC C2/C18 SC 2.1/10 column (3 μm; 2.1 mm × 100 mm). The peptides were eluted by a linear gradient of acetonitrile in 0.065% trifluoroacetic acid at a flow rate of 100 μl/min. The eluate was monitored at 215, 254 and 280 nm, and peptides were automatically collected using the peak-fractionation function. Selected peptides were subjected to automated amino acid sequence analysis in an Applied Biosystems model 477A peptide sequencer, equipped with an on-line phenylthiohydantoin amino acid analyser, model 120A. The instruments were operated according to the instructions of the manufacturer.

Saturation experiments

Saturation experiments were carried out to determine the apparent dissociation constant (K_d) for [^{3}H]oleic acid to purified E-FABP. In a typical assay, 3.3 μg of E-FABP in 50 μl of homogenization buffer [containing 0.3% gelatin as carrier (Bio-Rad)] was incubated overnight at 4 °C in the presence of increasing concentrations (2.4–12000 nM) of [^{3}H]oleic acid (10 Ci/mmol) in 8 mm × 40 mm glass microtubes. A 5 μl portion was taken to assess the total amount of radiolabelled ligand in the solution, and a 40 μl sample was analysed by the PAGE/radiobinding technique to measure the amount of bound ligand [17]. Scatchard-plot analysis was performed and apparent K_d calculated as described previously [23]. Saturation experiments were performed in three independent experiments and the K_d values obtained differed by less than 10%.

Preparation of polyclonal antibodies

Purified FABP (70 μg) was mixed with Freund's complete adjuvant and injected subcutaneously at multiple points on the back of a rabbit. At 3-week intervals after the initial injection, the rabbit was injected twice with 70 μg of FABP mixed with Freund's incomplete adjuvant and antiserum was collected 3 weeks after the second booster injection. Immune serum was stored at −70 °C until use.

The purity of the antibodies was checked by protein immunoblotting after separation of protein extracts by SDS/PAGE (15% gels) and PAGE (7.5% gels). Immunological detection was performed using the rabbit anti-(human E-FABP) serum at a dilution of 1:1000 and peroxidase-labelled goat anti-rabbit IgG Fab′ fragment (Cappel) at a dilution of 1:8000 as secondary antibody. The immunoreactive bands were visualized using diaminobenzidine and H_2O_2 as substrates.

Isoelectrofocusing

To determine the pI of native FABP, isoelectrofocusing studies were performed on horizontal slab polyacrylamide gels. The gels contained 5.8% acrylamide, 3.1% bisacrylamide, 10% sucrose and 6.3% ampholytes (Pharmacia), pH interval 3–10. Electrode solutions were 0.04 M aspartic acid (anode) and 1 M NaOH (cathode).

The purified E-FABP (2.5 μg) and protein samples (20–100 μg) from various epidermal cells were applied to the gel with a plastic applicator (Pharmacia). After focusing, the proteins were electrotransferred to a nitrocellulose sheet, as described for PAGE/autoradioblotting. E-FABP bands were detected by immunostaining with antiserum to E-FABP.

Immunohistochemistry

Punch biopsies from normal and psoriatic skin were obtained from the buttock. The patients with chronic psoriatic lesions received no topical or systemic treatment at the time of the biopsy. The samples were snap-frozen, and 4 μm cryosections were treated for 30 min with 0.3% H_2O_2 to abolish intrinsic peroxidase activity. Immunostaining was performed using E-FABP antiserum at a dilution of 1:200 and an avidin–biotin complex kit (Vectastin, Vector Laboratories, Burlingame, CA, U.S.A.). Staining was performed with diaminobenzidine.

RESULTS

Levels of E-FABP in epithelial tissues

Protein extracts from normal human skin, psoriatic skin, psoriatic scales and cultured undifferentiated and differentiating keratinocytes were incubated with 1.2 μM of [^{3}H]oleic acid (sufficient concentration to saturate the binding sites) and subsequently analysed by PAGE/autoradioblotting to detect E-FABP (Figure 1). All samples, except for normal skin (lane 1), displayed a significant radioactive band at R_F 0.34 which corresponds to E-FABP [14]. The radioactive band from psoriatic scales (lane 3) was completely abolished by an excess of non-labelled oleic acid (lane 4) demonstrating that oleic acid reversibly binds E-FABP. When [^{3}H]retinoic acid (lane 5) was used instead of [^{3}H]oleic acid, a radioactive band corresponding to CRABP-II [17] was detected demonstrating that E-FABP is specific for fatty acid binding. As the E-FABPs detected in scales were shown to have the same migration and binding properties as those detected in other epidermal cells, we concluded that the E-FABPs in these samples are identical. The levels of E-FABP in various samples increased as follows: normal skin < undifferentiated keratinocytes (lane 7) < differentiating keratinocytes (lane 6) < psoriatic lesions < psoriatic scales. Quantitative analysis of E-FABP by PAGE/radiobinding gave comparable results (Table 1). The second radioactive band with R_F 0.67 co-migrates with albumin, used as standard [14,17]. This band also almost completely disappeared in the presence of an excess of unlabelled ligand. The free [^{3}H]oleic acid moved with the front of the gel.

Purification procedure

As psoriatic scales contain the highest amounts of E-FABP and are easy to obtain, they were used as a source for further

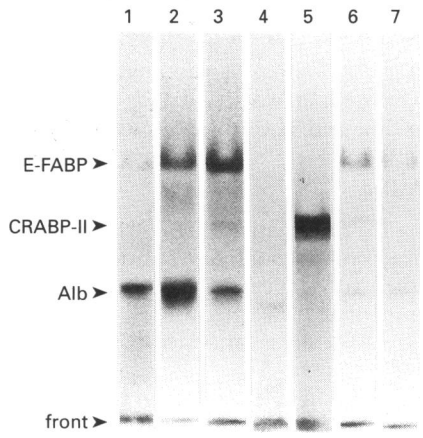

Figure 1 PAGE/autoradioblotting of [^{3}H]oleic acid-binding proteins from various human epithelial cells

Protein supernatants (300 μg) were incubated with 1.2 μM [^{3}H]oleic acid before PAGE analysis. After electrophoresis, the proteins were transferred to nitrocellulose sheets and radioactive bands were revealed by autoradiography (see the Experimental section). The band migrating at the front of the gel corresponds to excess free [^{3}H]oleic acid, whereas the Alb band shows identical mobility with human albumin. Lane 1, skin; lane 2, psoriatic lesions; lane 3, psoriatic scale; lane 4, psoriatic scale in the presence of a 500-fold excess of unlabelled oleic acid; lane 5, psoriatic lesions incubated with [^{3}H]retinoic acid revealing CRABP-II used as a control for specificity; lanes 6 and 7, cultured differentiating and undifferentiated keratinocytes respectively.

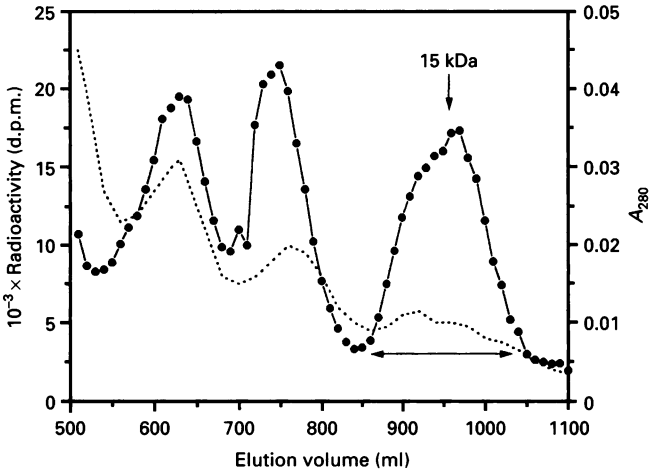

Figure 2 Elution profile of psoriatic-scale protein extract subjected to Sephadex G-100 chromatography

The protein sample from psoriatic scales (143 mg in 11 ml) was incubated with 1 μM [^{3}H]oleic acid and fractionated on a Sephadex G-100 column as described in the Experimental section. The material under the peak indicated was collected, concentrated and further purified by semipreparative PAGE. ●, Radioactivity; · · · · ·, A_{280}.

purification of proteins. The protein supernatant obtained from psoriatic scales was incubated with [^{3}H]oleic acid before being subjected to gel filtration on Sephadex G-100 (Figure 2). Three radioactive peaks were eluted from the column. The radioactive peak corresponding to 15 kDa which was eluted between 850 and 1050 ml was collected and concentrated. The progress of E-FABP purification was monitored by detection of a protein band at R_F 0.34 in polyacrylamide gel stained with Coomassie Blue. Because E-FABP shows electrophoretic mobilities distinct from

Table 1 Levels of E-FABP in human epidermal cells

E-FABP was measured by PAGE/radiobinding. The results are means ± S.D. of three experiments.

	E-FABP (pmol/mg of protein)
Human skin	
Normal	< 0.5
Psoriatic	42.5 ± 3.4
Cultured human keratinocytes	
Undifferentiated	3.4 ± 0.24
Differentiating	6.6 ± 0.53

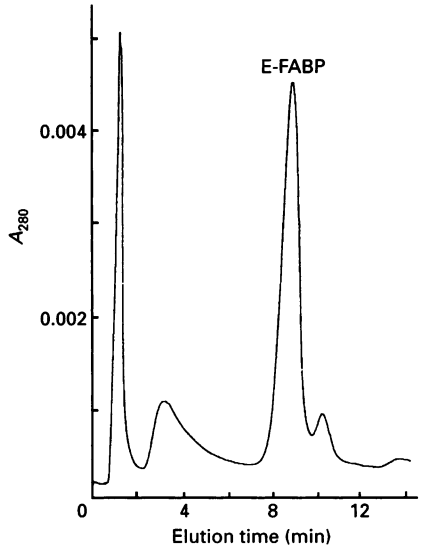

Figure 3 Elution profile of proteins obtained after semipreparative PAGE subjected to ion-exchange chromatography on Mono Q

Proteins (1.68 mg) eluted from semipreparative polyacrylamide gels were purified by h.p.l.c. on a Mono Q column equilibrated with 20 mM Tris/HCl buffer at pH 8. Elution of proteins was achieved using a gradient of 0–0.5 M NaCl in the same buffer. Two major protein peaks were eluted. E-FABP appeared at 8.8 min.

those of the proteins from adipose tissue, heart, liver and intestine (R_F 0.43, 0.45, 0.51 and 0.53 respectively; deduced from ref. [14] and unpublished results), the PAGE technique was used to isolate E-FABP on a semipreparative scale. To remove low-molecular-mass contaminants present in the E-FABP protein fraction extracted from the band obtained by semipreparative PAGE, it was further purified using ion-exchange chromatography (Mono Q). As shown in Figure 3, the E-FABP eluted at 8.8 min was pure (Figure 4), as only one band could be detected on SDS/PAGE and PAGE analysis (Coomassie Blue staining).

Properties of E-FABP

A saturation curve at equilibrium for purified E-FABP was obtained using increasing amounts of [^{3}H]oleic acid (Figure 5). Scatchard-plot analysis revealed a straight line indicating a single class of binding site. The apparent dissociation constant was 0.46 μM. The number of binding sites saturated with [^{3}H]oleic acid was about 5.2 times lower than the expected number

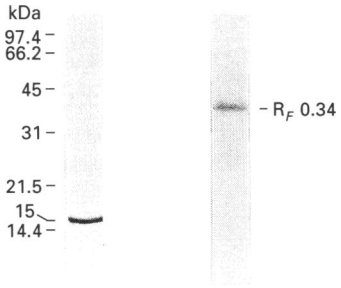

Figure 4 Analysis of E-FABP purity

E-FABP (5 μg) obtained by h.p.l.c. was analysed by SDS/PAGE (15% gel; left lane) and PAGE (7.5% gel; right lane). Proteins were stained with Coomassie Blue. E-FABP was considered homogeneous on the basis of the presence of only one band in both systems.

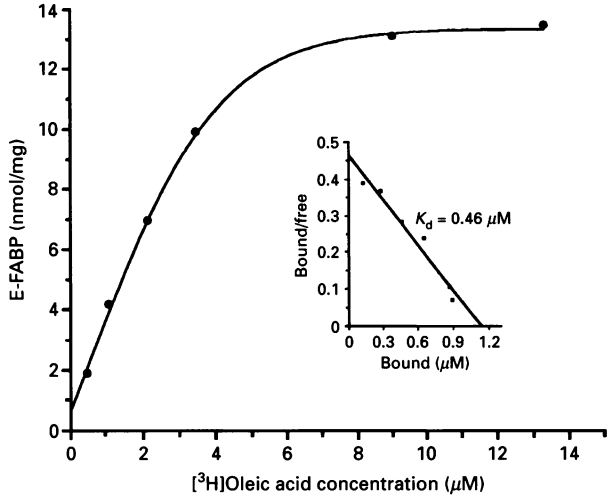

Figure 5 Scatchard-plot analysis of [^{3}H]oleic acid binding to E-FABP

A saturation curve at equilibrium was obtained when purified E-FABP (3.3 μg) was incubated with increasing amounts of [^{3}H]oleic acid. E-FABP–[^{3}H]oleic acid complex was measured by the PAGE technique as described in the Experimental section. The Scatchard plot (inset) was calculated on the basis of the saturation curve.

(assuming one binding site per E-FABP molecule) of total equivalent binding sites of the added E-FABP. These results suggest that either oleic acid at 13 μM is insufficient to compete with a putative endogenous high-affinity ligand bound to E-FABP or the E-FABP has partially lost its binding capacity during the purification process. The difference between the intrinsic protein content and the Lowry values might also account for the low number of binding sites (see below). The release of the ligand from E-FABP during PAGE, which could render the calculated K_d questionable, can be discounted for the reasons discussed previously [17]: the absence of a smear from the E-FABP radioactive band precludes the release of significant amounts of ligand; serum retinol-binding protein with similar K_d shows no retinol release with an identical technique. Furthermore, the use of the PAGE technique was found to be interesting for ligand-binding studies with retinoid-binding proteins as it greatly reduces non-specific co-aggregation of ligand with the protein core [17].

Various radiolabelled ligands were tested for their capacity to bind to purified E-FABP using a PAGE/radiobinding assay. From Table 2, we can deduce that C_{18} fatty acids show the highest affinity for E-FABP, but introduction of double bonds diminishes the binding. Decreasing the carbon chain length to C_{16} also reduces the affinity of the fatty acid for E-FABP. Squalene, cholesterol and 12-HETE, as well as retinoids and their isomers, showed no binding at all, suggesting that E-FABP binds fatty acids in a highly specific manner.

Table 2 Binding capacities of purified E-FABP for various ligands

Purified E-FABP (0.48 μM) in 50 mM Tris/HCl, pH 7.5, was incubated with the ligand being tested (1.2 μM). Binding capacities were measured by PAGE/radiobinding assay. Results are means of two experiments which did not differ by more than 12%.

Labelled ligands	Relative binding capacity (%)
Stearic acid, 18:0	100
Linoleic acid, *cis,cis*-18:2(*n*−6)	84.3
Oleic acid, *cis*-18:1(*n*−9)	61.8
Linolenic acid, all-*cis*-18:3(*n*−3)	18.1
Palmitic acid, 16:0	3.0
Arachidonic acid, all-*cis*-20:4(*n*−6)	1.0
Squalene	0
Cholesterol	0
12-HETE	0
all-*trans*-, 9-*cis*-13-*cis*-Retinoic acid	0
all-*trans*-Retinol	0

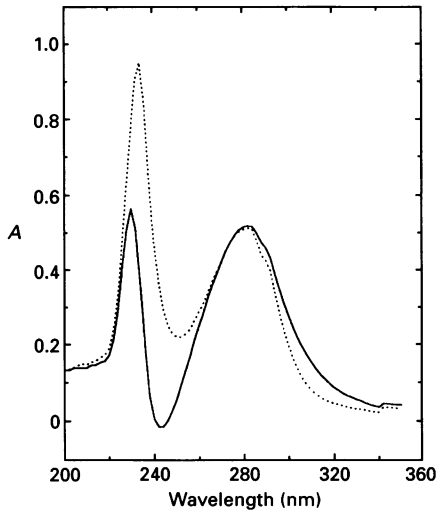

Figure 6 U.v.-absorption spectra of E-FABP

Delipidated E-FABP (——) and untreated E-FABP (····) were present at concentrations of 21.2 and 95.2 μM respectively. Spectra were obtained in PBS buffer, pH 7.4.

Spectral properties

Absorbance of purified E-FABP was maximal at 283 and 239 nm (Figure 6). No additional peak was observed near 350 nm, as seen for CRABP and CRBP, which could be due to a retinoid ligand [24,25]. Delipidation of E-FABP solution with hexane did not affect absorbance at 283 nm but the absorbance at 239 nm was reduced and shifted 4 nm towards the blue. Fluorescence excitation and emission spectra (uncorrected) of E-FABP are shown in Figures 7(a) and 7(b) respectively. The fluorescence emission was maximal at a wavelength of about 334 nm. The

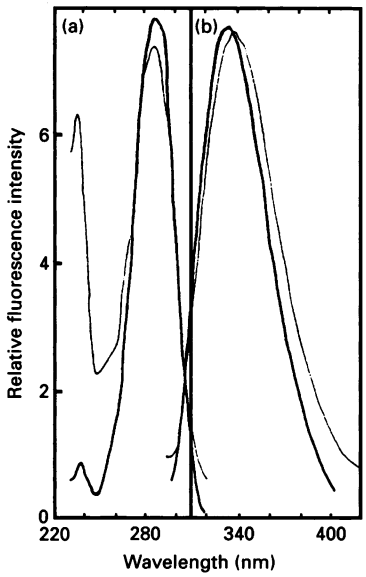

Figure 7 Uncorrected fluorescence spectra of E-FABP and apo-E-FABP

Excitation spectra (**a**) were determined with emission at 340 nm. Emission spectra (**b**) were determined with excitation at 280 nm. The Figure shows the spectrum of apo-E-FABP (5.1 μM) (——) and E-FABP (8.2 μM) (——) in PBS.

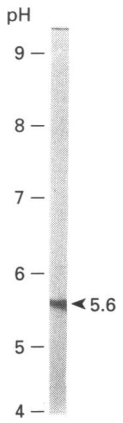

Figure 8 Isoelectrofocusing/PAGE/immunoblotting of psoriatic-scale protein extract

Proteins (20 μg) were analysed by horizontal isoelectrofocusing/PAGE using a 3–10 pH range system. After isoelectrofocusing, proteins were transferred to a nitrocellulose sheet and immunoreactive bands were revealed with E-FABP antibody as described in the Experimental section. pH values in the gel are indicated. They were measured with a pH-meter in gel slices placed in distilled water.

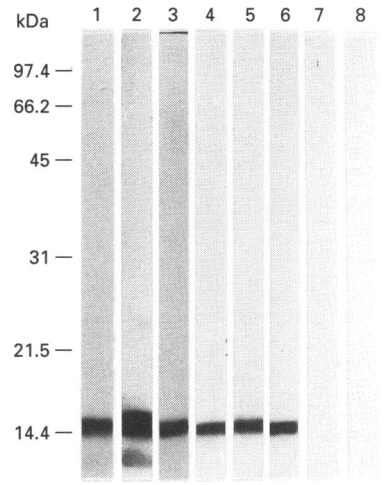

Figure 9 SDS/PAGE immunoblotting of protein extracts from various human tissues

Proteins (50 μg) were separated by SDS/PAGE (15% gels), and transferred to a nitrocellulose sheet. E-FABP bands were revealed with rabbit antiserum to E-FABP. Lane 1, skin; lane 2, psoriatic scale (20 μg); lane 3, purified E-FABP (2 μg); lane 4, intestine; lane 5, heart; lane 6, adipose tissue; lane 7, liver; lane 8, kidney. The positions of molecular-mass standards (kDa) are indicated on the left.

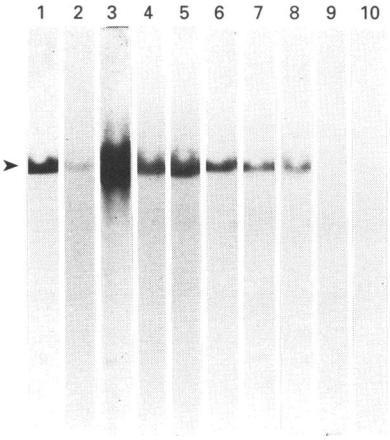

Figure 10 PAGE/immunoblotting of protein extracts from various human tissues

Proteins (300 μg) were separated by non-denaturing PAGE (7.5% gels), and transferred to a nitrocellulose sheet. E-FABP bands were revealed with rabbit antiserum to E-FABP. Lane 1, purified E-FABP (2 μg); lane 2, skin; lane 3, psoriatic lesions; lane 4, intestine; lane 5, heart; lane 6, adipose tissue; lanes 7 and 8, cultured differentiating and undifferentiated keratinocytes respectively; lane 9, liver; lane 10, kidney.

Stokes shift of 51 nm and the broad emission bandwidth may be consistent with at least a tryptophan in contact with water [26]. The excitation spectrum (Figure 7a) was maximal near 286 nm with a weak peak at 237 nm. Apo-E-FABP spectral analysis revealed a pattern similar to that seen for E-FABP, except for the peak at 237 nm which was higher.

Partial amino acid sequencing of E-FABP

The peptides obtained by cleavage of approx. 30 μg of FABP with LysC-specific protease were isolated by reversed-phase chromatography. The material under two peaks was sequenced: peak K1, Ile/Glu-Tyr/Ser-Glu/Thr-Lys/Ile-Thr-Arg-Lys; peak K2, Cys-Thr-Leu-Gly-Glu-Lys. Although sharp and symmetrical, peak K1 represented non-homogeneous material, which could, however, easily be identified as consisting of two peptides derived from LysC-specific protease digestion when compared with the published sequence of PA-FABP [15].

Isoelectrofocusing

Microheterogeneity of purified FABP due to possible isoforms, protein degradation or ligand effect has been reported by others

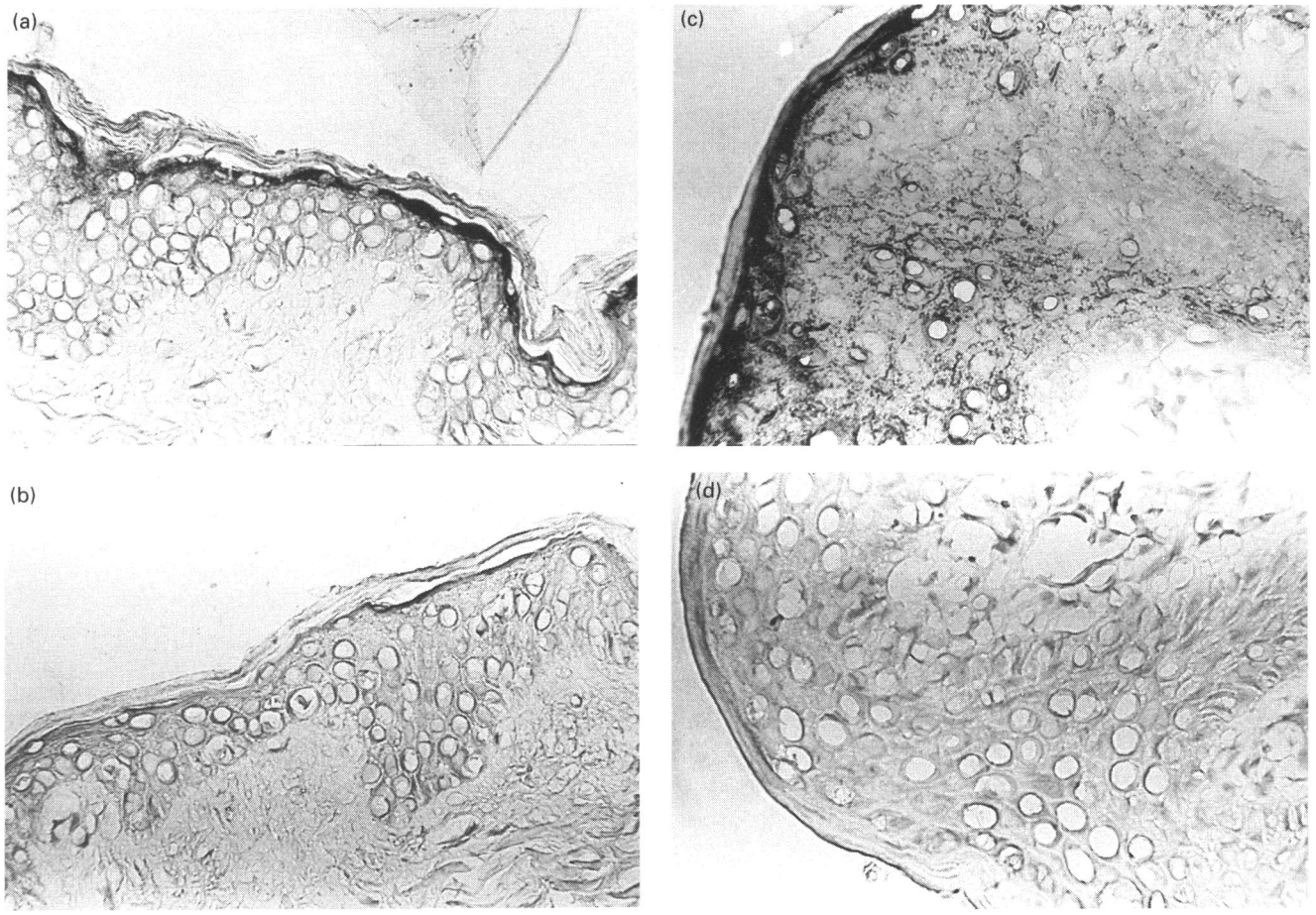

Figure 11 Immunohistochemical localization of E-FABP in human normal skin and in psoriatic lesions

Sections of frozen normal skin (a, b) and psoriatic lesions (c, d) were stained with antiserum to E-FABP (a, c) using the ABC technique as described in the Experimental section. Controls were stained with preimmune serum (b, d).

(see review in ref. [1]). Analysis of protein extract from psoriatic tissue by isoelectrofocusing/PAGE/immunoblotting showed the presence of only one immunoreactive band at pI 5.6 (Figure 8) and no detectable isoforms. This pI, which corresponds to that of the native structure of E-FABP, differs from the calculated pI of 6.96 for PA-FABP [15]. Identical results were obtained for purified E-FABP (not shown).

Expression of E-FABP in various tissues

Crude protein extracts from various human tissues were tested for the presence of E-FABP. Irrespective of the technique used (non-denaturing isoelectrofocusing/PAGE, PAGE/immunoblotting or denaturing SDS/PAGE/immunoblotting), only one immunoreactive band of similar mobility was detected in all samples studied, confirming E-FABP homogeneity at R_F 0.34. The absence of other immunoreactive bands with higher R_F values in PAGE/immunoblotting experiments demonstrates that E-FABP antiserum did not cross-react with FABP from intestine (0.53), liver (0.51), adipose tissue (0.43) or heart (0.45) (R_F deduced from ref. [14] and unpublished results). In similar experiments, antiserum to FABP from human heart and liver (generously provided by Professor J.-H. Veerkamp) showed no immunoreactivity with epidermal cell extracts but showed a specific immunoreactive band at R_F 0.45 and 0.51 for heart and liver protein supernatants respectively; these R_F values are identical with those observed using PAGE/autoradioblotting [14]. The molecular mass of E-FABP determined by SDS/PAGE/immunoblotting was similar in the various tissues studied. The fast-migrating band seen for psoriatic scales (Figure 9) (lane 2) might be due to E-FABP degradation products. The relative amounts of E-FABP in various tissues (Figure 10) decreased as follows: psoriatic lesion > heart > intestine > adipose tissue > cultured differentiating keratinocytes > cultured undifferentiated keratinocytes. No detectable amounts of E-FABP were found in liver and kidney.

Localization of E-FABP in normal human epidermis and psoriatic lesions

The presence and local distribution of E-FABP during normal (Figures 11a and 11b) and abnormal epidermal differentiation (psoriasis) (Figures 11c and 11d) were analysed using immunohistochemical techniques. Cytosolic expression of E-FABP was localized mainly in the stratum granulosum of normal skin (Figure 11a), whereas in psoriatic skin the staining was localized in the suprabasal layers with some strong labelling just below the horny layers. Interestingly, the basal layer was not stained. Staining with preimmune serum was negative for both normal

(Figure 11b) and psoriatic (Figure 11d) skin. No E-FABP could be detected in the dermis of either normal or psoriatic skin.

DISCUSSION

We describe the isolation, purification and characterization of human E-FABP which we recently detected in human epidermal cells [14]. E-FABP is a cytosolic protein, and displays similar characteristics to FABPs from other tissues with respect to molecular mass (15 kDa), K_d (0.46 μM) and binding properties for fatty acids. However, the pI (5.6) for E-FABP differs markedly from that of liver (6.0), heart (5.1), myelin and adipose tissue (9.0) [1]. In addition, the amounts of E-FABP found in cultured epidermal cells and skin samples (0.065% of total soluble proteins) were much lower than the FABPs found in heart, intestine and adipose tissue (about 5%) [2,4]. The scarcity of E-FABP in epithelial cells may explain the contradictory reports from various investigators with regard to its presence or absence [14]. An absorption peak near 350 nm, which is due to retinoid binding, is absent from the absorption spectrum of purified E-FABP, suggesting that the ligand of E-FABP does not participate in retinoid metabolism. Spectral analysis showed that the occupancy of the binding site by the endogenous ligand induces only small conformational changes in E-FABP, as shown by the 6 nm shift to the blue and the quenching of the 235 nm excitation peak. The similarity of the sequence of two peptides of E-FABP to the deduced sequence of the cDNA clone of PA-FABP [15] suggests that these two proteins might be identical. Ion-exchange chromatography and electrophoretic analysis of FABPs originating from different tissues demonstrated the existence of charge isoforms [1]. In contrast, isoelectrofocusing/immunoblotting and PAGE/immunoblotting showed no heterogeneity in E-FABP charge.

Binding experiments showed that approximately one-quarter of the expected number of binding sites of non-delipidated E-FABP were occupied by the ligand under saturation conditions, suggesting that E-FABP might also be present in an inactive form. This hypothesis is supported by the observation that the radioactive peak of E-FABP measured in the PAGE/radiobinding assay showed much lower amounts compared with the intensity of Coomassie Blue staining on PAGE. It seems therefore that inactive E-FABP is generated during the purification procedure. Phosphorylation of adipocyte FABP involved in signal transduction considerably diminishes ligand-binding affinity [27], suggesting that E-FABP could also be post-translationally modified, resulting in a physiologically inactive form. E-FABP possesses only one binding site and shows no binding affinity for cholesterol or its precursor squalene. It showed a high specificity for fatty acids, as neither retinol nor retinoic acid and its isomers bind to it. This is in contrast with the retinoid-binding properties observed for myelin and adipocyte FABP [1].

There is strong evidence that E-FABP may be involved in keratinocyte differentiation. Normal human skin contains very low amounts (< 0.5 pmol/mg of protein), localized almost entirely in the stratum granulosum. In abnormal differentiation of the epidermis (psoriasis), 42.5 ± 3.4 pmol of E-FABP/mg (approximating to the amounts found for CRABP-II [17]) were measured and found to be localized throughout the suprabasal cell layers, whereas staining was absent in the basal cell layer where the keratinocytes are still not committed to terminal differentiation [28]. Furthermore, during Ca^{2+}-induced keratinocyte differentiation, E-FABP levels were twice as high as those measured in undifferentiated keratinocytes.

In the psoriatic epidermis, the overexpression of E-FABP in suprabasal layers and the absence of staining in basal layers supports the theory of an alteration of fatty acid metabolism in this disease [29,30]. Studies of structure–ligand-binding relationships revealed that stearic acid has the highest affinity for E-FABP, and that decreasing the number of carbons or introducing double bonds into the fatty acid chain reduces the affinity of the ligand. Interestingly, the fatty acid species 18:0, 18:1 and 18:2 showed the highest affinity for E-FABP. Therefore the fatty acids of the C_{18} series, except for the $C_{18:1}$ species whose affinity is too low, might be transported by E-FABP. Sphingolipids are prominent components of cellular membranes, lipoproteins and other lipid-rich structures whose levels significantly increase during epidermal differentiation [12]. The fatty acids esterified to sphingolipids belong to the long-chain weakly saturated fatty acid series. Thus one may hypothesize that E-FABP–long-chain fatty acid complexes are involved in the synthesis of sphingolipids. Epidermal cells, normal and psoriatic, possess a high capacity for the metabolic transformation of arachidonic acid to 12-HETE [29,31]. It is rather unlikely that E-FABP is implicated in the transport of these ligands because of low, or absence of, affinity. This suggests that E-FABPs are not involved downstream of the metabolic crux of the arachidonic acid pathway. Because fatty acids are a major energy source in heart and skeletal muscle, heart FABPs are thought to transport fatty acids to mitochondria for oxidation [1]. The co-presence in low amounts of E-FABP besides heart, adipocyte and intestinal FABPs in their respective tissues strongly suggests a more specific role for E-FABP, unconnected with energy production or the formation of the skin lipid barrier, a system requiring high lipid synthesis [12].

The findings that saturated as well as unsaturated fatty acids transactivate the peroxisome proliferator-activated receptors [32], in addition to the low levels of E-FABP, prompt us to propose that E-FABP could regulate the intracellular levels of the ligand interacting with these receptors and therefore play a role similar to that reported for CRABPs. An E-FABP endogenous ligand with an affinity for peroxisome proliferator-activated receptors remains to be identified.

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REFERENCES

1 Veerkamp, J. H., Peeters, R. A. and Maatman, R. G. H. J. (1991) Biochim. Biophys. Acta **1081**, 1–24
2 Veerkamp, J. H., van Kuppevelt, T. H. M. S. M., Maatman, R. G. H. J. and Prinsen, C. F. M. (1993) Prostaglandins, Leucotrienes Essent. Fatty Acids **49**, 887–906
3 Sweetser, D. A. Heuckeroth, R. O. and Gordon, J. I. (1987) Annu. Rev. Nutr. **7**, 337–359
4 Börchers, T. and Spener, F. (1994) Curr. Top. Membr. **40**, 261–294
5 Offner, G. D., Brecher, P., Sawlivich, W. B., Costello, C. E. and Troxler, R. F. (1988) Biochem. J. **252**, 191–198
6 Börchers, T., Hojrup, P., Nielsen, S. U., Roepstorff, P., Spener, F. and Knudsen, J. (1990) Mol. Cell. Biochem. **98**, 127–133
7 Suzuki, M., Kitamura, K., Sakamoto, Y. and Uyemura, K. (1982) J. Neurochem. **39**, 1759–1762
8 Lowe, J. B., Boguski, M. S., Sweetser, D. A., Elshourbagy, N. A., Taylor, J. M. and Gordon, J. I. (1985) J. Biol. Chem. **260**, 3413–3417
9 Chan, L., Wei, C. F., Li, W. H., Yang, C. Y., Ratner, P., Pownall, H., Gotto, A. M., Jr. and Smith, L. C. (1985) J. Biol. Chem. **260**, 2629–2632
10 Sweetser, D. A., Birkenmeier, E. H., Klisak, I. J., Zollmann, S., Sparkes, R. S., Mohandas, T., Lusis, A. J. and Gordon, J. I. (1987) J. Biol. Chem. **262**, 16060–16071
11 Baxa, C. A., Sha, R. S., Buelt, M. K., Smith, A. J., Matarese, V., Chinander, L. L., Boundy, K. L. and Bernlohr, D. A. (1989) Biochemistry **28**, 8683–8690
12 Elias, P. M. and Menon, G. K. (1991) Adv. Lipid Res. **24**, 1–26

13 Chapkin, R. S., Ziboh, V. A. and Marcelo, C. L. (1986) J. Lipid Res. **27**, 945–954
14 Siegenthaler, G., Hotz, R., Chatellard-Gruaz, D., Jaconi, S. and Saurat, J.-H. (1993) Biochem. Biophys. Res. Commun. **190**, 482–487
15 Madsen, P., Rasmussen, H. H., Leffers, H., Honoré, B. and Celis, J. E. (1992) J. Invest. Dermatol. **99**, 299–305
16 Siegenthaler, G. (1990) Methods Enzymol. **189**, 299–307
17 Siegenthaler, G., Tomatis, I., Chatellard-Gruaz, D., Jaconi, S., Eriksson, U. and Saurat, J.-H. (1992) Biochem. J. **287**, 383–389
18 Rheinwald, J. G. and Green, H. (1975) Cell **6**, 331–334
19 Siegenthaler, G., Saurat, J.-H. and Ponec, M. (1990) Biochem. J. **268**, 371–378
20 Siegenthaler, G. and Saurat, J.-H. (1991) in Retinoids: 10 Years On (Saurat, J.-H., ed.), pp. 56–68, Karger, Basel
21 Siegenthaler, G., Saurat, J.-H. and Ponec, M. (1988) Exp. Cell Res. **178**, 114–126
22 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
23 Scatchard, G. (1949) Ann. N. Y. Acad. Sci. **51**, 660–692
24 MacDonald, P. N. and Ong, D. E. (1987) J. Biol. Chem. **262**, 10550–10556
25 Ong, D. E. and Chytil, F. (1978) J. Biol. Chem. **253**, 4551–4554
26 Nemecz, G., Jefferson, J. R. and Schroeder, F. (1991) J. Biol. Chem. **266**, 17112–17123
27 Buelt, M. K., Shekels, L. L., Jarvis, B. W. and Bernlohr, D. A. (1991) J. Biol. Chem. **266**, 12266–12271
28 Watt, F. M. (1984) J. Cell Biol. **98**, 16–21
29 Hammarström, S., Hamberg, M., Samuelsson, B., Duell, E. A., Stawiski, M. and Voorhees, J. J. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 5130–5134
30 Yardley, H. J. and Summerly, R. (1981) Pharmacol. Ther. **13**, 357–383
31 Hammarström, S., Lindgren, J. A., Marcello, C., Duell, E. A., Anderson, T. E. and Voorhees, J. J. (1979) J. Invest. Dermatol. **73**, 180–183
32 Göttlicher, M., Widmark, E., Qiao, L. and Gustafsson, J.-A. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 4653–4658

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