

Calcium-binding protein S100A7 and epidermal-type fatty acid-binding protein are associated in the cytosol of human keratinocytes

Gerry HAGENS, Isabelle MASOUYÉ, Eric AUGSBURGER, Raymonde HOTZ, Jean-Hilaire SAURAT and Georges SIEGENTHALER¹

Clinique de Dermatologie and DHURDV, Hôpital Universitaire, CH-1211 Geneva, Switzerland

Expression of epidermal-type fatty acid-binding protein (E-FABP) and S100A7 has previously been shown to be elevated in psoriatic skin, a disease characterized by abnormal keratinocyte differentiation. However, no causal relationship between the up-regulation of these proteins and the disease has been shown. E-FABP is thought to be involved in cytosolic fatty acid (FA) transport, whereas the role of S100A7 is still unknown. In this report, we show by overlay assays that E-FABP, immobilized on nitrocellulose, is able to capture S100A7 from cytosolic psoriatic protein extracts and vice versa, suggesting the formation of a complex between the two proteins. Using purified E-FABP and S100A7, the complex can be reconstituted only in presence of EDTA. Moreover, we show that increased EDTA concentrations in psoriatic cytosolic protein extracts enhance complex for-

matation. Partial complex disruption was obtained by the addition of physiological concentrations of Zn²⁺ (0.1 mM), whereas Ca²⁺ at 5 mM and Mg²⁺ at 30 mM had no effect. On the other hand, high Ca²⁺ concentrations (30 mM) resulted in partial complex disruption. Oleic acid-binding properties were observed for free E-FABP and the complex E-FABP–S100A7, but not for free S100A7. By using confocal microscopy we show that S100A7 and E-FABP are co-localized in the cytoplasm of differentiating keratinocytes from lesional psoriatic skin. These data indicate that formation of the E-FABP–S100A7 complex and its FA-binding function might be regulated at least by bivalent cations.

Key words: calcium-binding proteins, fatty acid-binding proteins, protein–protein interactions, psoriasis, zinc.

INTRODUCTION

The process of epidermal differentiation and stratification is controlled by a calcium gradient, increasing from the basal layer to the last living cell layer (stratum granulosum) [1]. During stratification, an increased lipid metabolism occurs in parallel with keratinocyte differentiation. Certain lipid metabolites become constituents of the skin-lipid barrier [2]. Disruption of this barrier accelerates lipid synthesis and alters the Ca²⁺ gradient in a reversible manner [3,4]. These observations suggest that fatty acid (FA) transport/metabolism might be related to Ca²⁺-dependent mechanisms involved in keratinocyte differentiation.

Intracellular FA transport in human epidermis is performed by specific carriers, such as the epidermal-type fatty acid-binding protein (E-FABP) [5–7] and the recently described FA–p34 complex composed of the Ca²⁺-binding proteins (CaBPs) S100A8 and S100A9 [8]. The fact that E-FABP and FA–p34 are highly overexpressed in lesional psoriatic skin [8–10] is in line with the discovery of an altered epidermal Ca²⁺ gradient [11] and an altered FA transport/metabolism associated with this disease [12–14]. S100A7, a CaBP of unknown biological function, is weakly expressed in the granular layer of normal skin, whereas it is highly expressed in the cytosol and the particulate fraction of psoriatic epidermis [15–17]. The seemingly co-ordinated up-regulation of E-FABP and S100A7 in psoriasis, and the fact that FA–p34 can bind FA in a Ca²⁺-dependent way, led to the hypothesis that the functions of E-FABP and S100A7 might be connected. In line with the general hypothesis concerning cross-talk between S100 proteins and FA transport/metabolism is the

finding that the generation of free FAs and lysophospholipids by cytosolic phospholipase A₂ might be controlled directly by p11, a member of the S100 protein family [18].

In this report, we describe a cross-talk mechanism mediated by bivalent cations between the CaBP S100A7 and the FA-transport protein E-FABP in the cytosol of differentiating cultured keratinocytes and psoriatic scales.

MATERIALS AND METHODS

Cell culture and tissue isolation

Normal human keratinocytes from foreskins were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Basel, Switzerland) containing 1.3 mM Ca²⁺ and 10% (v/v) fetal-calf serum [19]. After stratification, differentiating keratinocytes were separated from non-differentiated keratinocytes by the low-Ca²⁺-switch method, as described previously [20]. Psoriatic scales were obtained by gentle scraping of lesional skin from psoriatic patients as volunteers. Normal human skin was obtained from patients undergoing reconstructive surgery and lesional psoriatic human skin was obtained from diagnostic biopsies. Harvested cells and tissues were kept frozen at –20 °C until use.

Preparation of protein extracts

Tissues or cells were homogenized in Tris buffer [50 mM Tris/HCl/25 mM NaCl/2.5 mM EDTA/1 mM dithiothreitol (pH

Abbreviations used: CaBP, calcium-binding protein; CRABP-II, cellular retinoic acid-binding protein type II; E-FABP, epidermal-type fatty acid-binding protein; FA, fatty acid; IEF, isoelectric focusing.

¹To whom correspondence should be addressed (e-mail siegenth@cmu.unige.ch).

7.5)] using a Polytron tissue homogenizer, and centrifuged for 30 min at 100 000 *g* at 4 °C. The supernatant, referred to as the cytosolic fraction, was concentrated using centrifugal micro-concentrators (Amicon, Geneva, Switzerland) with a molecular mass cut-off of 3000 Da. Protein solutions were either used directly or stored at -20 °C. Protein concentrations were estimated using the Bio-Rad protein assay kit (Bio-Rad, Glattburg, Switzerland) with BSA as the standard. Homogeneous E-FABP and S100A7 proteins were obtained as described previously [6,16,17].

Co-purification of E-FABP, S100A7 and the E-FABP-S100A7 complex

The cytosolic fraction (275 mg) from psoriatic scales was subjected to gel filtration on a Sephadex G-100 column (4.5-cm × 100 cm) (Pharmacia LKB, Uppsala, Sweden). The column was equilibrated with 5 mM sodium phosphate buffer containing 0.2 M NaCl, 2 mM dithiothreitol, 1 mM PMSF and 0.02% (v/v) sodium azide at pH 7.5, and calibration was performed using Blue Dextran, albumin, ovalbumin, chymotrypsin, myoglobin and the human E-FABP-[³H]oleic acid complex. The column was eluted at a flow rate of 0.8 ml/min, and the fractions eluting at a molecular mass around 15 kDa [6] were pooled and dialysed overnight against 20 mM imidazole/HCl, pH 6.0. Proteins were concentrated as described above, and 7.0 mg was loaded on to a cation-exchange column (Resource S; Pharmacia), equilibrated with the dialysis buffer. The column was connected to a HPLC system (Varian, Basel, Switzerland). Proteins were eluted in a linear gradient using equilibration buffer containing up to 0.5 M NaCl. Two major peaks were eluted. Since most of E-FABP was found within peak I from 1 to 5 min, fractions containing this peak were pooled, dialysed overnight in 20 mM Tris/HCl, pH 8.0, and concentrated as described above. Concentrated proteins were loaded on to an anion-exchange column (Resource Q; Pharmacia) equilibrated with the Tris/HCl buffer, pH 8.0, and proteins were eluted with the same buffer containing 0.5 M NaCl in a linear gradient. E-FABP-containing fractions were pooled, concentrated, dialysed against Tris/HCl buffer, pH 8.0, and loaded on to a second anion-exchange column, as described above. Proteins were eluted as previously described, and all eluted fractions were subjected to by SDS/PAGE and SDS/PAGE immunoblotting for the presence of E-FABP and S100A7.

Immunoblotting and immunoprecipitations

The cytosolic fractions from various samples were separated by SDS/PAGE (15% gels) and subsequently transferred on to a nitrocellulose membrane (Electran, BDH Laboratory Supplies, U.K.). The membrane was blocked with PBS containing 5% (w/v) skimmed dry milk. After blocking, the membrane was incubated in PBS containing 0.5% skimmed dry milk, 0.2% (v/v) Tween 20 and antisera directed against E-FABP [6] or S100A7 [17] (each at a dilution of 1:1000). Immunoreactive bands were detected using a horseradish-peroxidase-labelled goat anti-(rabbit IgG Fab') fragment (Cappel, Durham, NC, U.S.A.) and 3,3'-diaminobenzidine dihydrochloride (Sigma, Deisenhofen, Germany) and H₂O₂ as substrates. As described earlier, the antisera did not cross-react with other proteins [6,10,17,21].

For immunoprecipitations, 1 mg of the cytosolic fraction was pre-cleared for 2 h at 4 °C using an aliquot of the corresponding pre-immune serum and by adding half the volume of a 50% Protein A-Sepharose (Pharmacia) slurry in PBS. The sample was

centrifuged at 14 000 *g* for 15 min and the supernatant was transferred to a new tube. Pre-cleared protein extract was incubated for 2 h at 4 °C with either anti-(E-FABP) antiserum or an irrelevant rabbit IgG antiserum. Antibody-antigen complexes were incubated for 1 h at 4 °C with Protein A-Sepharose (as described above) before centrifugation. Pellets were washed with homogenization buffer containing 200 mM NaCl and 1% (v/v) Triton X-100, and subsequently resuspended in Laemmli's sample buffer and boiled for 5 min before separation by SDS/PAGE (15% gels). Separated proteins were processed for immunoblotting, as described above.

Overlay assay

Purified E-FABP, S100A7, S100A8, S100A9 and recombinant cellular retinoic acid-binding protein type II (CRABP-II) were obtained as described previously [6,8,16,22]. Each purified protein (5 µg) was spotted on to nitrocellulose strips. The strips were then blocked in PBS (without Ca²⁺ and Mg²⁺; Gibco BRL, Basel, Switzerland) containing 5% (w/v) skimmed dry milk, and subsequently incubated overnight at 4 °C with 3 mg of the cytosolic fraction of cultured human keratinocytes or psoriatic scales. Protein extracts were prepared as described above using Tris/HCl buffer containing 2.5 mM EDTA. To examine whether the immobilized proteins were able to capture E-FABP or S100A7 from protein extracts, the membranes were processed for immunodetection using the corresponding antisera, as described above. A manuscript describing the anti-CRABP-II antiserum is in preparation (results not shown).

Isoelectric focusing (IEF)

Partially purified samples (15 µg) were obtained by separating the pooled 15 kDa fractions from the Sephadex G-100 column (see above) by PAGE (7.5% gels). The strong Coomassie-Blue-stained band migrating at an *R_F* of 0.34 (also see Figure 3, upper panel), which consists mainly of E-FABP and S100A7 [6,16,17], was excised and proteins were extracted. Extracted proteins were diluted in isourea buffer (65 mM dithioerythritol/65 mM CHAPS/9 M urea) and separated in a precast Immobiline gel (Immobiline Drystrips; Pharmacia) containing a non-linear pH gradient of 3-10, as according to the manufacturer's instructions. The run was performed with a voltage increasing linearly from 300 V to 3500 V in 3 h, followed by a constant voltage of 3500 V to give a total of 100 kVh. After IEF, the Immobiline gel strips were stained with Coomassie Blue. pI values were defined using pI standards (Bio-Rad).

Complex reconstitution and disruption

For reconstitution experiments, E-FABP and S100A7 (2.4 µg each, obtained as described previously [6,16]) were mixed in the absence or presence of increasing concentrations of EDTA dissolved in twice-distilled water at pH 7.5. The samples were incubated overnight at 4 °C, or for 2 h at room temperature. For disruption experiments, cytosolic protein extracts were dialysed against 50 mM Tris/HCl, pH 7.4. Dialysed protein extracts (400 µg) were subsequently incubated overnight at 4 °C with MgCl₂, CaCl₂, ZnCl₂, EDTA, or in combinations thereof, at the indicated concentrations. After incubation, proteins were separated by PAGE (7.5% gels) and the gel was subsequently stained with Coomassie Blue. The stained band migrating at an *R_F* of 0.34 was excised, and proteins were extracted from the gel slice as described previously [6,17]. Extracted proteins were separated by SDS/PAGE (15% gels) and subsequently blotted on to a

nitrocellulose membrane. The membrane was processed for immunodetection, as described above.

Oleic acid-binding assays

The partially purified complex (0.5 and 4 mg of non-delipidated protein), corresponding to the 15 kDa fractions from the Sephadex G-100 column, was incubated for 2.5 h at room temperature with 1 μ M [3 H]oleic acid ([9,10- 3 H]oleic acid, 10 Ci/mmol; DuPont New England Nuclear, Boston, MA, U.S.A.) as described previously [5]. Bound radioligand was separated from free ligand using charcoal-dextran, as described previously [23]. The mixture was injected into an ion-exchange Mono-Q HR5/5 column (Pharmacia), and proteins were eluted as described for the anion-exchange column. The protein elution profile was monitored by UV absorbance at 280 nm, and radioactivity was assessed in a liquid-scintillation counter by counting the radioactivity in 100 μ l portions of each eluted fraction in 4 ml of Ultima Gold (Packard, Zürich, Switzerland). For assays using S100A7 or E-FABP alone, either 15 or 5 μ g of purified proteins (non-delipidated) respectively were incubated with 1 μ M [3 H]oleic acid for 2.5 h at room temperature, before injection into a Superose 12 column (Pharmacia). Elution of proteins was performed with PBS containing 0.2 M NaCl at a flow rate of 0.8 ml/min. After a delay of 8 min, fractions of 0.4 ml were collected for determination of bound radioactivity.

Immunohistochemistry and confocal microscopy

Tissue samples were fixed for at least 24 h in 10% (v/v) formalin and processed for routine histology. Paraffin sections (5 μ m) were used for immunostaining by using standard protocols, except that sections were permeabilized in 0.3% (v/v) Triton X-100 solution for 2 \times 10 min at room temperature. Non-specific binding sites were blocked by preincubating the sections with normal goat serum. After incubation for 1 h at room temperature with either the anti-(E-FABP) or anti-S100A7 antiserum (dilution 1:200), an immunoperoxidase technique using biotinylated goat anti-(rabbit IgG) as a secondary antibody and a horseradish-peroxidase-conjugated avidin-biotin complex was applied following the manufacturer's instructions (Vectastain reagents, Vector Laboratory, Burlingame, CA, U.S.A.). Peroxidase activity was revealed using H₂O₂ and diaminobenzidine as the chromogenic substrate. The sections were slightly counterstained with haematoxylin. As a negative control, the pre-immune serum from the immunized rabbit was used simultaneously on adjacent sections. For confocal microscopy, the anti-(E-FABP) antiserum was complexed with anti-(rabbit IgG) coupled to rhodamine at dilutions of 1:200 and 1:40 respectively in PBS. The anti-S100A7 antiserum was complexed with anti-(rabbit IgG) coupled to FITC using the same dilutions as above. Sections were preincubated twice for 20 min in PBS containing 0.5% (w/v) BSA and 0.3% (v/v) Triton X-100, and these were then incubated for 2 h at room temperature with the anti-(E-FABP)-anti-(rabbit IgG) complex. After two 10 min washes in PBS, sections were incubated for 2 h with the anti-S100A7-anti-(rabbit IgG) complex, and subsequently washed twice for 10 min in PBS, before microscopical analysis was performed with a Zeiss LSM 410 apparatus. FITC-derived fluorescence was detected at 488 nm (excitation wavelength) and over the range 510–525 nm (for emission), whereas rhodamine-derived fluorescence was detected at 543 nm (excitation wavelength) and over the range 580–630 nm (for emission).

RESULTS

Co-purification of E-FABP, S100A7 and the E-FABP-S100A7 complex

Since E-FABP and S100A7 are highly overexpressed in psoriatic scales, this sample was used for co-purification. In the first step, proteins from the cytosolic fraction were separated by gel filtration on a Sepharose G100 column. The E-FABP-containing fractions, which were eluted as a large asymmetric peak at about 15 kDa (results not shown; [6,17]), were pooled. This sample contained mainly S100A7 and E-FABP (Figure 1, upper right panel, lane A), and was subjected to cation-exchange chromatography (Resource S). Figure 1 (upper left panel) shows the protein-elution profile, consisting of two major peaks. The eluted fractions were analysed by SDS/PAGE and stained with Coomassie Blue (Figure 1, upper right panel). As confirmed by immunoblotting, most of the E-FABP was eluted in the first peak (1 to 6 min) and S100A7 was eluted essentially in the second peak (13 to 15 min). However, all fractions contained E-FABP and S100A7. Only fractions eluting from 1 to 5 min, containing E-FABP and S100A7 in significant amounts, were pooled and used for further association analysis. Coomassie Blue staining of the SDS/PAGE separated pooled fractions showed that the E-FABP content was now similar to the amount of S100A7 (Figure 1, lower right panel, lane A), suggesting an enrichment of E-FABP.

The pooled fractions were injected into an anion-exchange column (Resource Q). Figure 1 (lower left panel) shows the elution profile, and Figure 1 (lower right) the SDS/PAGE analysis of the eluted fractions. E-FABP and S100A7 were co-eluted in all fractions, although their amounts differed significantly in some fractions. When fractions containing both proteins were subjected to an additional anion-exchange chromatographic process, an almost identical elution profile was obtained, with fractions containing low amounts of virtually pure E-FABP and S100A7 (results not shown). These experiments indicate that E-FABP, S100A7 and an eventual E-FABP-S100A7 complex can be co-purified using ion-exchange chromatography. Similar results were obtained when protein extracts from cultured human keratinocytes were used (results not shown).

pI values of native S100A7 and E-FABP

In order to determine whether the co-purification of E-FABP and S100A7 is due to their having an identical pI value, E-FABP-containing fractions from gel filtration were separated by non-denaturing PAGE. As shown in earlier reports [6,16,17], the strong Coomassie-Blue-stained band migrating at an R_f value of 0.34 (see also Figure 3, upper panel) consisted mainly of E-FABP and S100A7. The band was excised, and the proteins were extracted from the gel. Proteins were then separated by IEF on an Immobiline gel containing a non-linear pH gradient of 3–10, and stained with Coomassie Blue. Three major protein bands were detected, excised from the IEF gel and subsequently treated with Laemmli's sample buffer, before analysis by SDS/PAGE immunoblotting. E-FABP was detected in the bands migrating at apparent pI values of 5.70 and 5.75, whereas S100A7 migrated with the band at an apparent pI value of 5.55 (calculated using IEF standards). These observations suggest that IEF dissociates the complex into three bands, and indicate the existence of at least two E-FABP populations. The pI values for E-FABP and S100A7 have previously been calculated using two-dimensional gel electrophoresis. The described values were obtained using denatured proteins from crude keratinocyte extracts, and differ

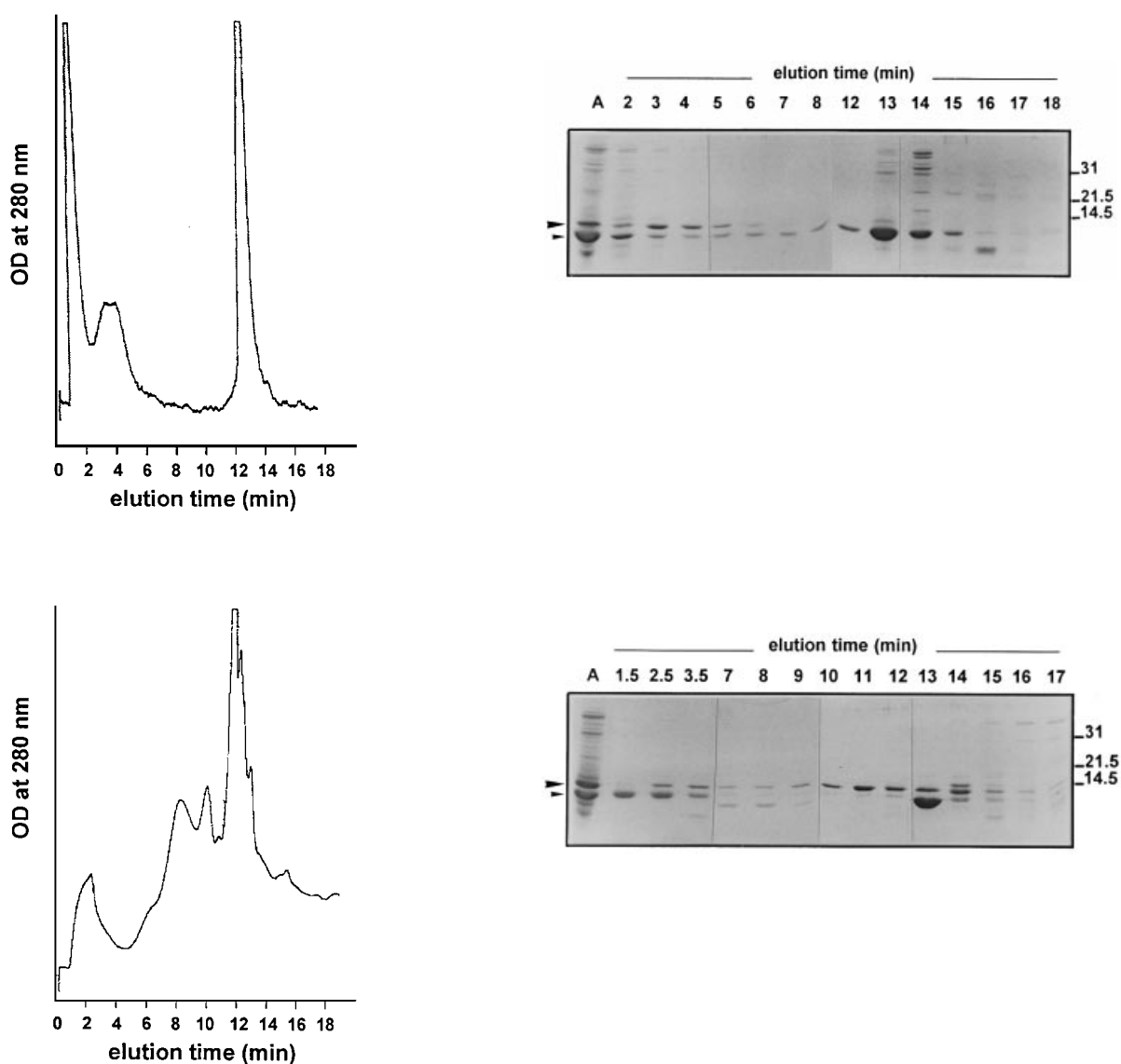


Figure 1 Co-purification of E-FABP and S100A7 by ion-exchange chromatography

Cytosolic proteins were subjected to serial ion-exchange chromatography, as described in the Materials and methods section. Upper left panel: elution profiles of proteins (after the gel-filtration step) separated by the Resource S column. Upper right panel: Coomassie-Blue-stained SDS/polyacrylamide gel of the corresponding fractions. Elution profile of proteins (pooled fractions from 1 to 5 min of the Resource S column) subjected to Resource Q chromatography is shown in the lower left panel, and the corresponding Coomassie Blue-stained SDS/PAGE analysis of eluted fractions is in the lower right panel. Thick and thin arrowheads indicate E-FABP and S100A7 respectively. Molecular mass indicators in kDa are shown on the right of the gels featured in the upper and lower right panels.

from those in the present study (i.e. 6.77 and 6.2 for S100A7 [15,24] and 6.3 for E-FABP [24]). However, besides experimental differences, it cannot be excluded that different isoforms of both proteins might co-exist in human keratinocytes.

Overlay assays

To obtain more information about a possible association of S100A7 with E-FABP, overlay assays were performed. Thereby, purified human proteins (E-FABP, S100A7 and recombinant S100A8, S100A9 and CRABP-II) were spotted on to nitrocellulose strips and, after blocking, incubated with the psoriatic cytosolic fraction. Binding of cytosolic CRABP-II, S100A7 or E-

FABP to one of the immobilized proteins was monitored using the specific antisera directed against CRABP-II, S100A7 and E-FABP respectively, as described elsewhere [6,17,21]. As shown in Figure 2, E-FABP on strip 2 was able to capture S100A7 from the cytosolic protein extract, as revealed with the specific antiserum directed against S100A7. On the other hand, strip 3, which was revealed with the anti-(E-FABP) antiserum, shows that immobilized S100A7 is able to associate with E-FABP from the cytosolic fraction, thus confirming the former experiment. Control experiments using keratinocytes in the highly abundant proteins S100A8 and S100A9 (highly abundant members of the S100 family) and CRABP-II, which belongs to the same family as E-FABP, showed no significant binding of E-FABP or S100A7

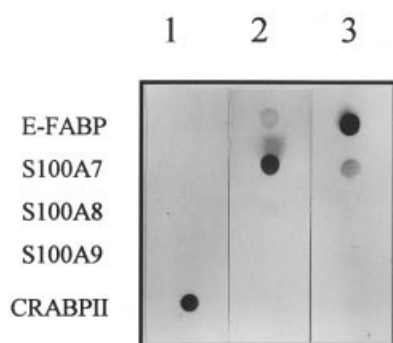


Figure 2 Overlay assays using purified proteins and cytosolic protein extracts

Purified E-FABP, S100A7, S100A8, S100A9 and recombinant CRABP-II ($5 \mu\text{g}$ each) were spotted on to three nitrocellulose strips (1–3). After blocking, each strip was incubated with 3 mg of cytosolic protein extracts from psoriatic scales. The strips were subsequently treated for immunodetection as described in the Materials and methods section. Strip 1: immunodetection using anti-(CRABP-II); strip 2: immunodetection using anti-S100A7; strip 3: immunodetection using anti-(E-FABP).

to these immobilized proteins, or vice versa (strip 1 and results not shown).

Complex reconstitution

The same amounts of purified E-FABP and S100A7 (obtained as described previously in [6,16]) were mixed in the presence or absence of increasing concentrations of EDTA, and incubated for 16 h at 4°C before analysis with non-denaturing PAGE. Coomassie Blue staining of the gel shows a band at an R_f of 0.34 (Figure 3, upper panel, lanes 1–4). The proteins in the various bands were extracted from the gel and analysed by SDS/PAGE immunoblotting using the antisera directed against E-FABP and S100A7 (Figure 3, lower panel). The data show the presence of S100A7 and E-FABP in the bands only in the presence of EDTA (lanes 2–4). The total Ca^{2+} level in the buffer was $150 \mu\text{M}$, as measured by Siegenthaler et al. [8], suggesting that S100A7–E-FABP complex reconstitution does not occur at such low Ca^{2+} concentrations. As a control, we show that S100A7 does not enter into the gel matrix in the absence or presence of EDTA (Figure 3, upper panel, lanes 5–8). The observations indicate that migration of S100A7 into the gel depends on binding to E-FABP, which occurs at low Ca^{2+} concentrations.

Studies on S100A7–E-FABP complex stability

In order to investigate whether bivalent cations might influence complex formation, cytosolic protein extracts from psoriatic scales were incubated with different concentrations of MgCl_2 , CaCl_2 and ZnCl_2 in the presence or absence of EDTA. Proteins were subsequently separated by non-denaturing PAGE, and the proteins in the single band at R_f 0.34 were analysed by SDS/PAGE immunoblotting with E-FABP and S100A7 antisera. As shown in Figure 4, increasing the Zn^{2+} concentration to up to 0.1 mM resulted in a significant disruption of the E-FABP–S100A7 complex (lane 5). Mg^{2+} and Ca^{2+} alone (lanes 2, 3, 6 and 7) or in combination (results not shown) had no obvious effect on the complex stability. On the other hand, EDTA (50 mM) alone, or in combination with Mg^{2+} or Ca^{2+} , increased

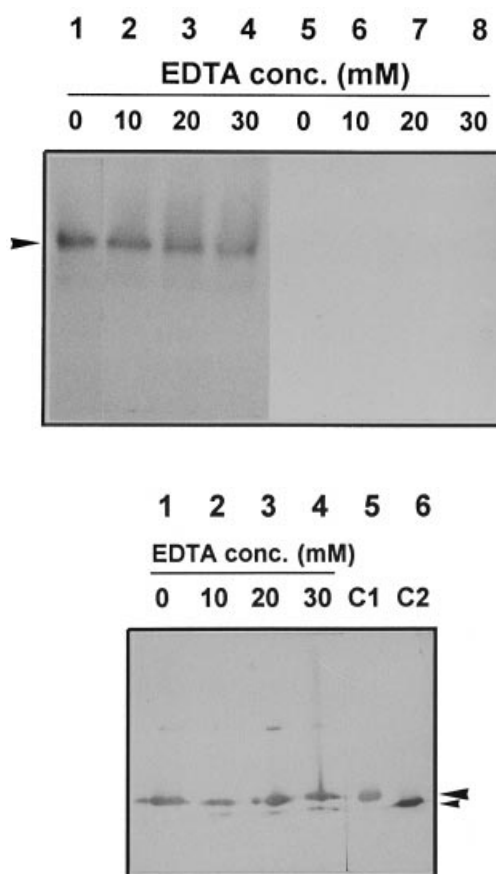


Figure 3 Co-migration and complex reconstitution using purified proteins

Purified E-FABP and S100A7 ($2.4 \mu\text{g}$ each) were mixed and incubated in the presence or absence of increasing amounts of EDTA, as described in the Materials and methods section. After incubation, samples were separated by non-denaturing PAGE (upper panel, lanes 1–4). As a control, S100A7 alone ($2.4 \mu\text{g}$) was analysed the same way (upper panel, lanes 5–8). S100A7 samples showed no Coomassie Blue staining, suggesting that S100A7 does not enter the gel matrix. Proteins migrating at R_f 0.34 (shown by the arrowhead) were extracted and analysed by SDS/PAGE immunoblotting. Immunodetection was performed simultaneously using anti-(E-FABP) and anti-S100A7 antisera. Lower panel: thick and thin arrowheads indicate E-FABP and S100A7 respectively; C1 and C2, purified E-FABP and S100A7 controls respectively.

complex formation as shown by the increased intensity of the S100A7 band (lanes 8, 9, 11, 13 and 15). EDTA even inhibited the Zn^{2+} -mediated complex disruption (lanes 14 and 15) suggesting that the zinc effect is reversible. Combinations of Zn^{2+} with the other cations had no synergistic effect on complex disruption (results not shown).

Preliminary results showed that S100A7 co-immunoprecipitated with E-FABP from cytosolic fractions using a specific antiserum directed against E-FABP [17]. In order to investigate whether co-immunoprecipitations are influenced by Ca^{2+} , this type of experiment was performed in the presence of various non-physiological Ca^{2+} concentrations. As shown in Figure 5, E-FABP could be immunoprecipitated from cytosolic protein extracts at about equal amounts, independent of the Ca^{2+} concentration. On the other hand, S100A7 co-immunoprecipitation decreased when Ca^{2+} concentrations were increased to 30 mM. These findings are specific, since neither E-FABP nor S100A7 were detected in the negative controls using an irrelevant rabbit antiserum (lanes N).

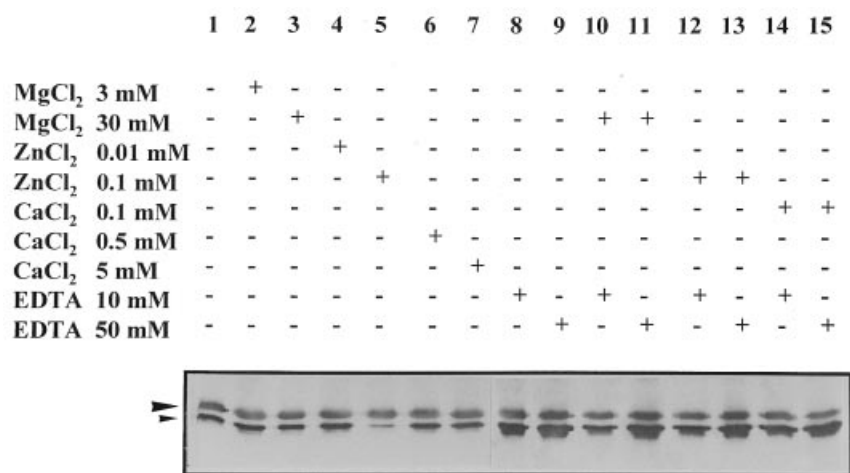


Figure 4 Disruption of the E-FABP–S100A7 complex by Zn²⁺

Cytosolic protein extracts were prepared as described in the Materials and methods section and either left untreated or incubated with the indicated concentrations of MgCl₂, CaCl₂ or ZnCl₂ in the presence or absence of EDTA. Protein extracts (0.4 mg) were subsequently separated by non-denaturing PAGE (7.5% gels). Proteins migrating at an *R_f*-value of 0.34 were extracted and analysed by SDS/PAGE immunoblotting. Immunodetection was performed simultaneously using anti-E-FABP and anti-S100A7 antisera. Thick and thin arrowheads indicate E-FABP and S100A7 respectively.

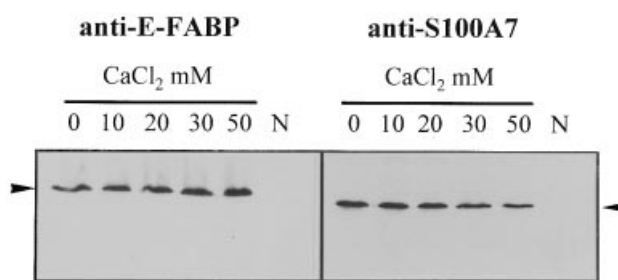


Figure 5 Co-immunoprecipitations of S100A7 with E-FABP in the presence or absence of Ca²⁺

Co-immunoprecipitations were performed as described in the Materials and methods section in the presence or absence of Ca²⁺. Precipitates were divided into two parts, separated by SDS/PAGE and subsequently blotted on to a nitrocellulose membrane for immunodetection with either anti-E-FABP or anti-S100A7 antisera. The thick or thin arrowhead indicates E-FABP or S100A7 respectively. N, a negative control using an irrelevant rabbit polyclonal antibody.

E-FABP still binds oleic acid when complexed with S100A7

Gel filtration is normally the appropriate method for ligand-binding studies; however, owing to the fact that free E-FABP, S100A7 and the E-FABP–S100A7 complex have too similar molecular masses, radiobinding assays were performed using ion-exchange chromatography. Partially purified S100A7–E-FABP complex (non-delipidated 15 kDa fractions obtained from gel filtration) was incubated with tritiated oleic acid before injection into a Mono Q column. Two injections with either 0.5 mg or 4 mg of complex were performed. The radioactive elution profiles revealed two well-defined peaks that were co-eluted with the protein-elution profiles (Figure 6, top panel). The heights of the radioactive peaks obtained with the 4 mg sample are approx. 8-fold greater than the heights of the corresponding peaks obtained with the 0.5 mg sample, demonstrating that these two peaks are in equilibrium and that protein loading on the column did not exceed the column capacity. The first radioactive

peak eluting between 1 and 3 min was constituted mainly of S100A7 and a smaller portion of E-FABP (Figure 6, centre panel) confirming observations described above (Figure 1c). The major part of E-FABP was eluted at about 6 min with a low amount of S100A7 compared with the first peak. Almost homogeneous S100A7 eluting between 15 and 16 min showed no binding of tritiated oleic acid (Figure 6, top). This was confirmed by radiobinding assays on a Superose 12 column using purified S100A7 (Figure 6, bottom panel). We conclude that E-FABP in the non-retained fractions is bound to S100A7, and that the E-FABP subunit of this complex is responsible for [³H]oleic acid-binding.

E-FABP and S100A7 are co-expressed in psoriatic skin and co-localize in the cytosol of psoriatic keratinocytes

In order to gain more information about the expression of S100A7 and E-FABP in normal and diseased human skin, immunohistochemistry and confocal microscopy were performed using antisera directed against the two proteins. In normal skin, S100A7 staining is confined to a few keratinocytes, from the outer part of the stratum granulosum (Figure 7a), whereas E-FABP is expressed in the uppermost stratum spinosum and in the stratum granulosum (Figure 7d).

In lesional psoriatic skin both proteins are cytosolic and highly overexpressed. In contrast to E-FABP (Figure 7e), S100A7 is also expressed in the basal layer (Figure 7b), where nuclear staining is also observed. The high specificity of the E-FABP antiserum has been shown previously [6]. The S100A7 antiserum is also specific, as shown by the absence of staining with the corresponding pre-immune serum (Figure 7c) and by lack of cross-reactivity in SDS/PAGE immunoblotting. Confocal microscopy was used to investigate the intracellular localization of both proteins. We show that S100A7 (Figure 7g), as well as E-FABP (Figure 7f), localize mainly in the cytosol of suprabasal keratinocytes from lesional psoriatic skin, as confirmed by double staining of the section (Figure 7h). In some cells, S100A7 and E-FABP also seem to be localized in the endoplasmic reticulum, as shown by the perinuclear staining. Certain cells show a bright nuclear staining for S100A7.

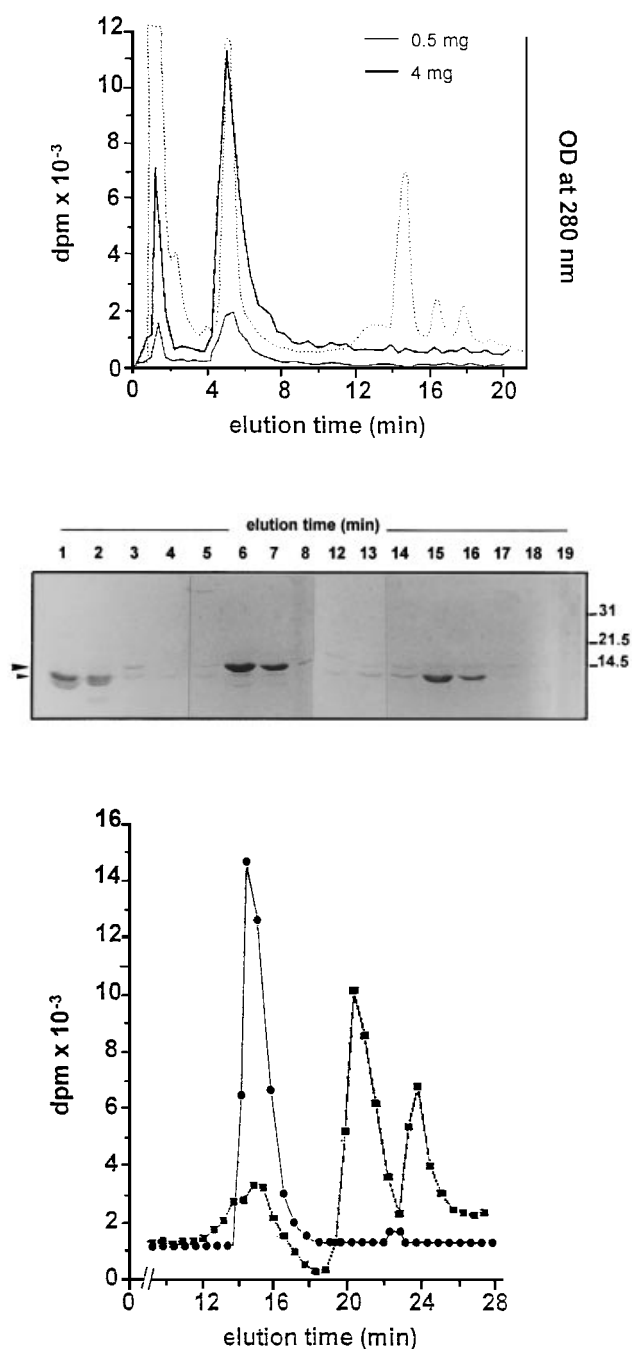


Figure 6 FA-binding property of the E-FABP-S100A7 complex

The partially purified complex was incubated with [3 H]oleic acid and analysed on a Mono Q column, as described in the Materials and methods section (top panel). A first injection was carried out with 0.5 mg of material. The two protein peaks were co-eluted with the two radioactive peaks. A second injection with 4 mg of protein confirmed the first experiment, suggesting that the first peak is not due to an overloading of the column. The dotted line represents the A_{280} (OD at 280 nm) for the 0.5 mg sample; the continuous lines show the elution profiles from the radioactivity experiment. The fractions were then analysed by SDS/PAGE for the presence of E-FABP and S100A7 (centre panel). The fractions eluting at 15 to 16 min on the ion-exchange column, possessing no [3 H]oleic acid-binding activity, constituted almost homogeneous S100A7. Thick and thin arrowheads indicate E-FABP and S100A7 respectively, and molecular mass indicators in kDa are shown on the right-hand side of the gel. To confirm that S100A7 possessed no FA-binding properties (bottom panel), 15 μ g of purified S100A7 (■) and 5 μ g purified E-FABP (●) were incubated with [3 H]oleic acid (1 μ M) before injection into a Superose 12 column. The elution profile from the radioactivity experiment in the bottom panel shows that E-FABP was eluted as a single peak at 15 kDa, whereas S100A7 showed no significant binding. The two radioactive peaks eluting after 20.5 min and 24 min correspond to unbound tritiated ligand.

DISCUSSION

In this work we have designed specific experiments to demonstrate and analyse the interaction between S100A7 and E-FABP [17].

Using both cation- and anion-exchange chromatography we show that portions of E-FABP and S100A7 remain associated when protein extracts of psoriatic scales are analysed by these techniques. Although E-FABP and S100A7 have distinct pI values, they migrate under specific conditions (PAGE) as a single band with an R_f value (0.34) identical with that of E-FABP alone, confirming previous results [16,17]. This finding, together with the fact that free S100A7, owing to its acidic properties, does not enter the polyacrylamide gel matrix, provides further evidence for the association of these two proteins. Direct evidence for the formation of an E-FABP-S100A7 complex was obtained by overlay assays, showing that immobilized E-FABP is able to capture S100A7 from the cytosolic fraction of psoriatic scales, and vice versa.

As reported previously [6,16,17], the separation of E-FABP and S100A7 is facilitated by the intermediate PAGE step. This might be explained by the fact that the excess of free S100A7 does not enter the polyacrylamide gel matrix, and is removed during PAGE. This, in turn, might disturb the equilibrium of E-FABP-S100A7 complex formation, resulting in an easier purification of E-FABP and S100A7.

The levels of complex formation under different conditions can be measured by analysing the ratio of the amount of E-FABP to that of S100A7 in the protein band at R_f 0.34. Thereby we show that association is increased in the presence of EDTA (up to 50 mM), indicating that association increases when the concentration of bivalent cations is decreased. Interestingly, physiological concentrations of Zn^{2+} (0.1 mM) in the protein extracts of psoriatic scales significantly decrease the level of the complex. Ca^{2+} at 5 mM and Mg^{2+} , even at 30 mM, have no influence on complex stability (even in the presence of other bivalent cations; results not shown). In contrast, high non-physiological levels of Ca^{2+} (30 mM) disrupt the complex, as shown by co-immunoprecipitation. These data indicate that Ca^{2+} and Zn^{2+} might have specific functions in regulating the stability of the complex. S100 proteins, including S100A7, bind Ca^{2+} , Zn^{2+} and Mg^{2+} reversibly on specific binding sites, and in a co-operative way [16,25–30]. The nature of the interaction between E-FABP and S100A7 is not known; however, it has been reported that binding of Ca^{2+} and Zn^{2+} induce conformational changes in S100 proteins [25–31]. Together with the fact that binding of Zn^{2+} to some S100 proteins induces a remarkable increase in the affinity for Ca^{2+} [26,32], this strongly suggests that Ca^{2+} and Zn^{2+} might co-operate in the control of E-FABP-S100A7 complex levels. Since S100 proteins contain one Ca^{2+} -binding site of high and one of low affinity [31], one might speculate that binding of Ca^{2+} to only one binding site (occurring at very low Ca^{2+} concentrations obtained in the presence of EDTA or in the absence of Zn^{2+}) has a different effect on complex formation/stability than binding of Ca^{2+} to both sites (occurring at high Ca^{2+} concentrations). Interestingly, Ca^{2+} and Zn^{2+} are important cations involved in the control of keratinocyte differentiation in the skin [33].

Our data show that E-FABP, S100A7 and the complex of E-FABP-S100A7 are in equilibrium in solution, and the formation of this complex is regulated by the levels of bivalent cations. This equilibrium hinders the determination of the exact stoichiometry of the complex. A first clue towards unravelling this is provided by gel filtration, where the shoulder (containing E-FABP and S100A7) of the asymmetric peak [6,17] represents protein eluting

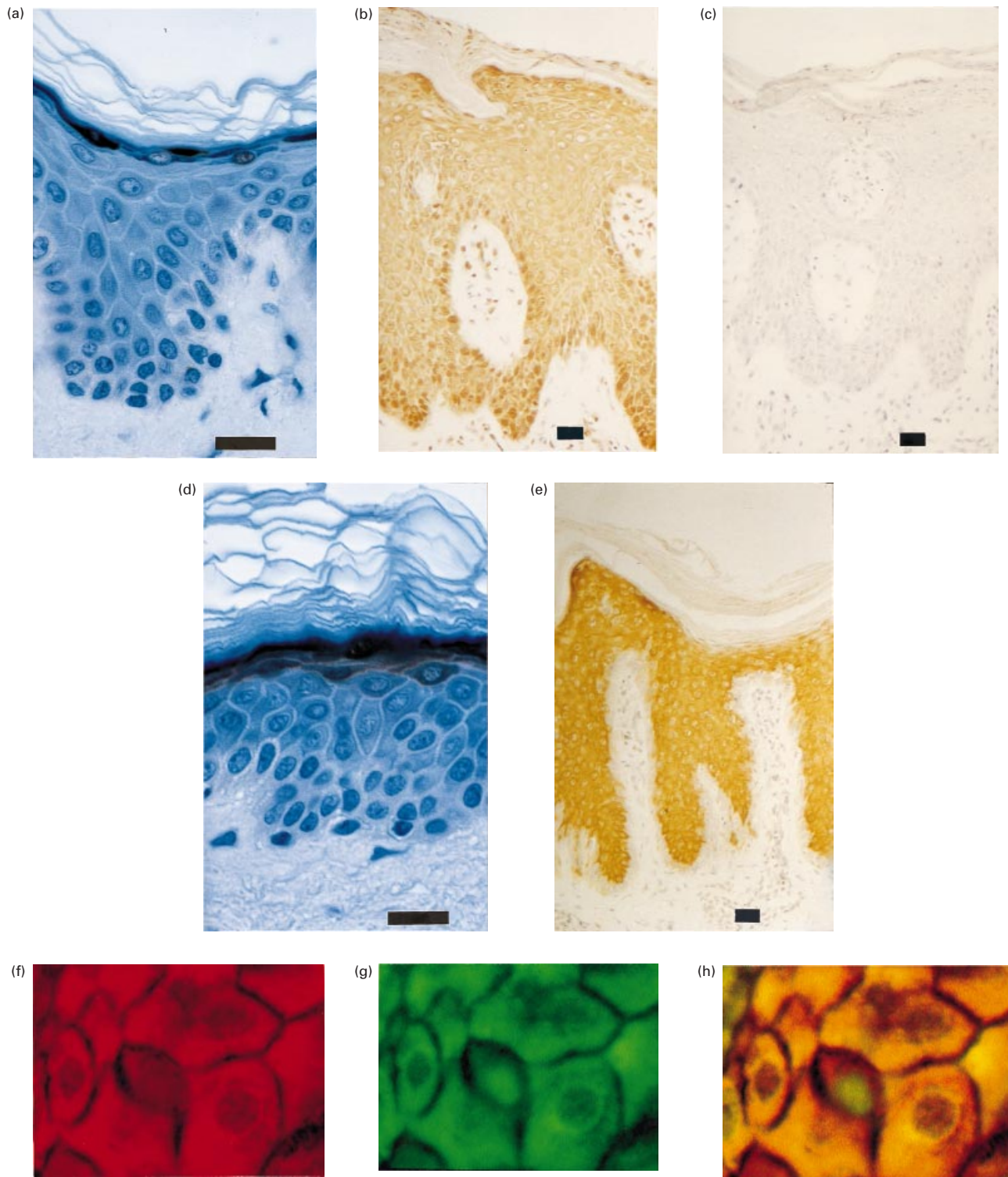


Figure 7 Localization of E-FABP and S100A7 in normal and diseased skin

Peroxidase-revealed immunohistochemical staining of S100A7 (**a** and **b**) and E-FABP (**d** and **e**) in normal (**a** and **d**) and lesional psoriatic skin (**b**, **c** and **e**). No staining was observed using the pre-immune serum of S100A7 as a control (**c**). Confocal microscopical analysis of E-FABP (**f**), S100A7 (**g**) and double staining (**h**) in lesional psoriatic skin. Scale bars represent 25 μm .

at a molecular mass of between 20 and 30 kDa, suggesting the presence of a heterodimeric form of the complex.

By radiobinding experiments, we show that E-FABP and the E-FABP-S100A7 complex have the capacity to bind FAs, whereas free S100A7 has no FA-binding properties. This indi-

cates that FAs are not only transported by free E-FABP, but probably also by the E-FABP-S100A7 complex.

Immunohistochemical analyses of lesional psoriatic skin show that E-FABP and S100A7 are co-expressed in all suprabasal cell layers. Both proteins are cytoplasmic, but in some cells S100A7

is also located in the nucleus. These data were confirmed by confocal microscopy, which also shows a perinuclear staining for S100A7 and E-FABP. Both perinuclear and nuclear S100A7 might account for the membrane-attached S100A7 described previously [16]. The fact that S100A7 is also expressed in the basal cell layer of lesional psoriatic skin, i.e. in the absence of any known FA-binding protein [5,6], indicates that both proteins are expressed independently. One might also speculate that any function of S100A7 depends on the availability of proteins interacting with S100A7. In this context, it has been reported that certain S100 proteins interact with basic helix-loop-helix transcription factors, and thereby inhibit them [34]. A similar function might also be attributed to S100A7, taking into account its nuclear localization in cells of the basal layer.

The intracellular Ca^{2+} concentration regulates differentiation of keratinocytes [35,36], which involves many different CaBPs [37,38]. The alteration of the extracellular Ca^{2+} gradient in psoriatic skin might be responsible for the up-regulation of these CaBPs, a group in which S100A7 is included. E-FABP is supposed to participate in differentiation-dependent lipid metabolism of keratinocytes [6,10]. The fact that S100A7 exists also as a membrane-attached form [16] makes it tempting to speculate that, in psoriatic keratinocytes, cytosolic S100A7 might serve as a docking protein for E-FABP, guiding it in a bivalent-cation-dependent fashion to sites where ligands might be available.

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