

PA-FABP, a novel marker of human epidermal transit amplifying cells revealed by 2D protein gel electrophoresis and cDNA array hybridisation

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Abstract Human epidermal stem cells express higher levels of $\beta 1$ integrins than their more differentiated daughters, transit amplifying cells. In a search for additional stem and transit cell markers we used proteomics and differential cDNA hybridisation to compare keratinocytes fractionated on the basis of $\beta 1$ integrin expression. There were remarkably few differences between the two populations and none of the RNAs differed in abundance by more than 2-fold. Nevertheless, proteomics revealed upregulated expression of epidermal fatty acid binding protein (PA-FABP, also known as E-FABP), Annexin II and two keratin related proteins in the transit population. An unknown high molecular mass protein was upregulated in the stem cell population. The upregulation of PA-FABP was confirmed by Northern blotting and conventional and whole mount labelling of human epidermis. We conclude that PA-FABP is a novel marker of epidermal transit amplifying cells. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Human epidermis is renewed throughout adult life by proliferation of a subpopulation of cells in the basal layer, known as stem cells [1,2]. Stem cells produce both stem and non-stem daughter cells. The non-stem daughters, known as transit amplifying cells, proliferate a small number of times and then undergo terminal differentiation as they move through the suprabasal epidermal layers to the tissue surface.

Although there are many markers that distinguish suprabasal from basal keratinocytes there are very few that distinguish stem from transit amplifying cells [2]. One such marker is expression of extracellular matrix receptors of the $\beta 1$ integrin family [3,4]. $\beta 1$ integrins are expressed by all cells in the basal layer of the epidermis, but keratinocytes with properties of stem cells have 2–3-fold higher levels than transit amplifying cells. $\beta 1$ integrin expression can be used to enrich for stem or transit amplifying cells, either from keratinocyte cultures or

direct from the epidermis, using FACS or differential adhesiveness to extracellular matrix proteins. Using confocal microscopy it is also possible to visualise the relative location of the different subpopulations [5]. The differences in integrin levels are functionally significant, because if a dominant negative integrin mutation is introduced into keratinocytes exit from the stem cell compartment is stimulated [6].

Approximately 10% of cells in the epidermal basal layer are thought to be stem cells, but the proportion of basal cells with high $\beta 1$ integrin expression varies between body sites and ranges from 25% to over 40% [1,2,4]. There is therefore a need for additional markers of stem and transit amplifying cells. We have used two complementary approaches to search for such markers. The first is to exploit the comprehensive database of human keratinocyte proteins that has been established using computer assisted two-dimensional (2D) gel electrophoresis ([7]; <http://biobase.dk/cgi-bin/celis>), as this allows the analysis of thousands of keratinocyte proteins simultaneously. Such comparisons of keratinocytes grown under different conditions have proven to be a powerful method for identifying disease and differentiation associated proteins [8]. In parallel, differential hybridisations of arrayed cDNAs derived from the UniGene human gene catalogue [9] allow for the analysis of the expression of approximately 30 000 ESTs representing different putative transcripts.

2. Materials and methods

2.1. Antibodies

For FACS and confocal microscopy, $\beta 1$ integrins were detected using mouse monoclonal antibody P5D2 [10]. PA-FABP was detected in epidermal sheets using a rabbit antiserum raised against whole PA-FABP referred to as anti-C-FABP (kindly provided by H. Fujii, Department of Biochemistry, Niigata University School of Medicine, Japan) [11,12]. Second layer antibodies used for immunostaining were Alexa 594 or FITC conjugated goat anti-rabbit IgG and Alexa 488 or FITC conjugated goat anti-mouse IgG (Molecular probes, Eugene, OR, USA).

2.2. Cell culture

Isolation of human keratinocytes from newborn foreskin and cultivation on a feeder layer of mitomycin C treated 3T3 cells have been described previously [3,4,6]. Second or third passage confluent keratinocytes (strains kp, kq) were used for protein and RNA analysis. The culture medium consisted of one part Ham's F12 medium and three parts Dulbecco's modified Eagle's medium, supplemented with 1.8×10^{-4} M adenine, 10% foetal calf serum, 0.5 μ g/ml hydrocortisone, 5 μ g/ml insulin, 10^{-10} M cholera toxin and 10 ng/ml epidermal growth factor.

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2.3. FACS and adhesion selection of keratinocytes

For FACS, keratinocytes were harvested with trypsin/EDTA and labelled with P5D2 and FITC conjugated secondary antibody, as described previously [3,4,6]. Propidium iodide labelling was used to gate out dead cells and suprabasal cells were gated out on the basis of their high forward and side scatter [3,4]. Basal cells were sorted on the basis of integrin expression on a Becton-Dickinson FACStar Plus. The 20% of basal cells expressing the lowest and highest levels of $\beta 1$ integrins were collected as representatives of transit amplifying and stem cell enriched populations, respectively [3,4]. The cells were immediately lysed and extracted for isolation of protein or RNA.

Cells were also fractionated on the basis of adhesion to 100 $\mu\text{g}/\text{ml}$ human type IV collagen, as described previously [3,4]. Cells that had adhered after 20 min constituted the stem cell enriched fraction.

Cells that were to be subjected to 2D protein gel electrophoresis were metabolically labelled prior to FACS or adhesion selection. Pre-confluent early passage primary human keratinocytes were incubated at 37°C for 18 h in methionine free Dulbecco's modified Eagle's medium containing 10% FCS supplemented with 200 $\mu\text{Ci}/\text{ml}$ [^{35}S]methionine (Amersham) and 2.4 mg/ml unlabelled methionine.

2.4. 2D gel electrophoresis

Keratinocyte proteins labelled with [^{35}S]methionine were extracted and subjected to 2D gel electrophoresis (IEF, NEPHGE) using methods described previously [13,14]. The first dimension was performed either as (i) IEF (18 h at 400 V) in 125×2 mm 4% polyacrylamide gels containing 2% w/v carrier ampholytes (1.6%, pH 5–7, Serva; 0.4%, pH 3.5–10, Pharmacia Biosystems AS) or (ii) NEPHGE (4.5 h at 400 V) in 125×2 mm 4% polyacrylamide gels containing 5% w/v carrier ampholytes (3% w/v, pH 7–9, Pharmacia Biosystems AS; 2% w/v, pH 8–9.5, Pharmacia Biosystems AS). 1D gels were applied to the second dimension and run at room temperature at 10 mA for 4 h and at 3 mA overnight. Between one and two million trichloroacetic acid precipitable counts were applied per gel. Following fluorography, the dried gels were exposed to X-ray film for 1–14 days.

2.5. Screening of cDNA arrays

Two sets of cDNA arrays were obtained from the Resource Centre for the German Human Genome Project (RZPD; www.rzpd.de) in Berlin [15]. 16 128 PCR products [9] from Image EST clones were selected by S. Haas, S. Kirby, M. Peters, B. Toussiant, H. Lehrach, A. Poustka, M. Vingron and B. Korn from the Unigene database and spotted in duplicate on a 22×22 cm nylon filter by the RZPD. The membranes were hybridised with probes obtained from poly(A)+ RNA prepared from $\beta 1$ integrin high and low expressing keratino-

cytes using the Dynabeads mRNA direct isolation kit (Dyna). Poly(A)+ RNA from 1–3 million cells was reverse transcribed in the presence of 100 μCi [^{33}P]dCTP (NEN, specific activity 3000 Ci/mmol) in a 100-fold excess of cold dCTP. Following the hybridisation and washing from 0.5×SSC, 0.1% SDS to 0.1×SSC, 0.1% SDS, the filters were exposed onto a phosphorimager screen (Molecular Dynamics) and scanned after 4 days. The images were analysed using the programs Xdigitise V3 (developed by Huw Griffiths; www.molgen.mpg.de) and Cgen developed by W. Lehrach. Bacterial stabs for Image clones of interest were obtained from the RZPD.

2.6. Northern blotting

Total keratinocyte RNA was extracted using Trizol (Gibco BRL). Approximately 1 μg total RNA was loaded on a 1% agarose gel containing 0.0175 M MOPS salt, 80 mM sodium acetate, 0.25 mM EDTA and 2 M formaldehyde and electrophoresed in the same concentration of MOPS. The RNA was blotted onto nylon membranes (Hybond N+, Amersham). Membranes were prehybridised, hybridised and washed using normal procedures. The bands were visualised either by autoradiography or exposure to a phosphorimager plate and subsequent scanning. A multiple tissue Northern blot was purchased from Clontech and hybridised as described above. The PA-FABP specific probe was the full length cDNA obtained from the RZPD (Image clone 772914).

2.7. Immunofluorescence staining of epidermis

8 μm frozen sections of neonatal human foreskin were briefly fixed in acetone, blocked for 30 min in PBS containing 0.2% v/v fish skin gelatin, then incubated with primary antibody followed by secondary antibody. Antibodies were diluted in PBS containing 0.2% fish skin gelatin. Sections were washed extensively in PBS after each antibody incubation.

Double labelling of whole mounts of epidermis from normal adult cheek skin was performed as described previously [5]. Formalin fixed epidermal sheets were simultaneously permeabilised and blocked by incubation in 0.5% skim milk powder, 0.25% fish skin gelatin (Sigma), 0.5% Triton X-100 in 0.9% NaCl, 20 mM HEPES, pH 7.2 (PB buffer) for 30 min. The primary antibodies were diluted in PB buffer and incubated at room temperature overnight with gentle agitation. The epidermis was then washed in PBS containing 0.2% Tween 20 for 3–4 h with several changes of wash buffer. The incubation with secondary antibodies was performed in the same way. A Zeiss 510 confocal microscope was used to analyse the whole mounts: 50–100 optical sections of each epidermal sheet were captured with an increment of 0.5–1.0 μm .

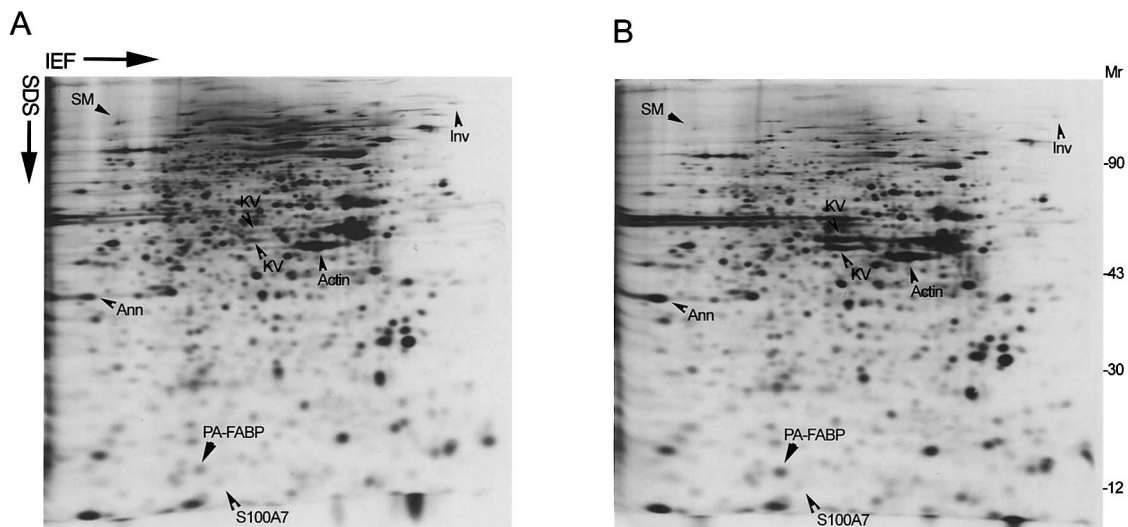


Fig. 1. 2D protein gel electrophoresis of [^{35}S]methionine labelled proteins extracted from basal keratinocytes expressing high (A) or low (B) levels of $\beta 1$ integrins. Positions of the following proteins are indicated by arrowheads: involucrin (Inv), unknown putative stem cell marker (SM), Annexin II (Ann), actin, PA-FABP, S100A7, keratin related proteins (KV). Positions of molecular mass markers (kDa) are indicated.

3. Results

3.1. Detection of differentially expressed proteins by 2D gel electrophoresis

Cultured human keratinocytes were incubated overnight in [35 S]methionine, then harvested, labelled with an antibody to β 1 integrins and subjected to FACS. After gating out dead cells and suprabasal cells, basal cells were fractionated into the 20% of cells with highest β 1 integrin levels (stem cell enriched) and the 20% of cells with lowest levels (transit amplifying cell enriched) [3,4]. The cells were extracted and subjected to 2D protein gel electrophoresis. A total of 10 inde-

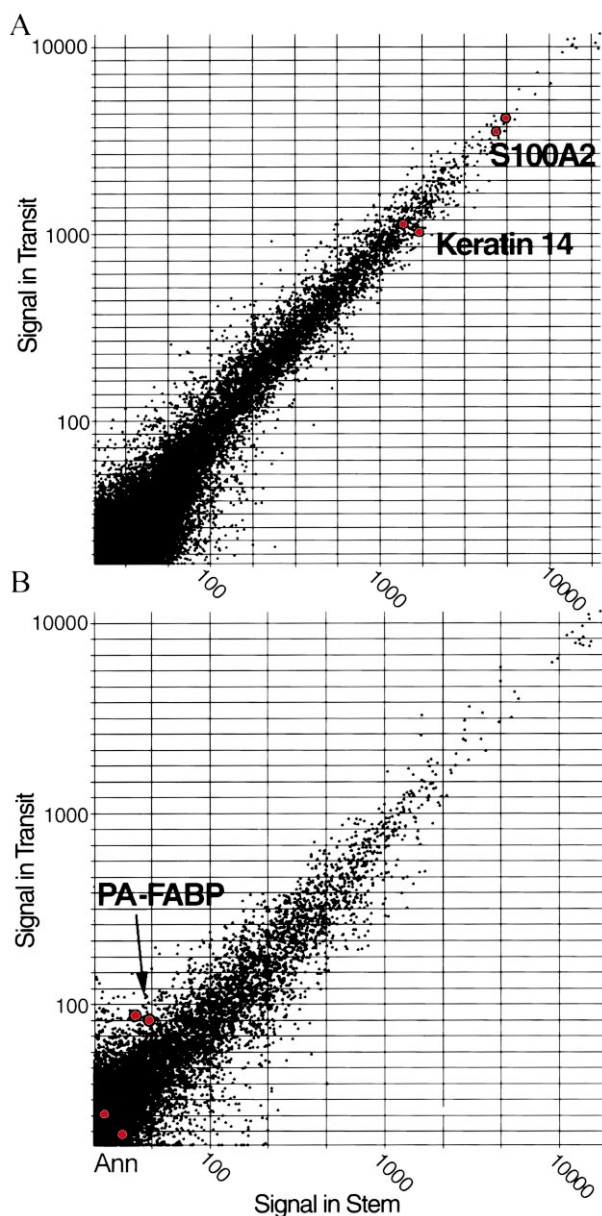


Fig. 2. Scatter profiles (from the Cgen program) of the average signals from hybridisation of cDNA extracted from keratinocytes expressing high (Stem) or low (Transit) levels of β 1 integrins to filters containing two different cDNA arrays (A and B respectively). Duplicate signals for S100A2, keratin 14, PA-FABP and Annexin II (Ann) are shown. The scales are the log of the normalised signal intensities.

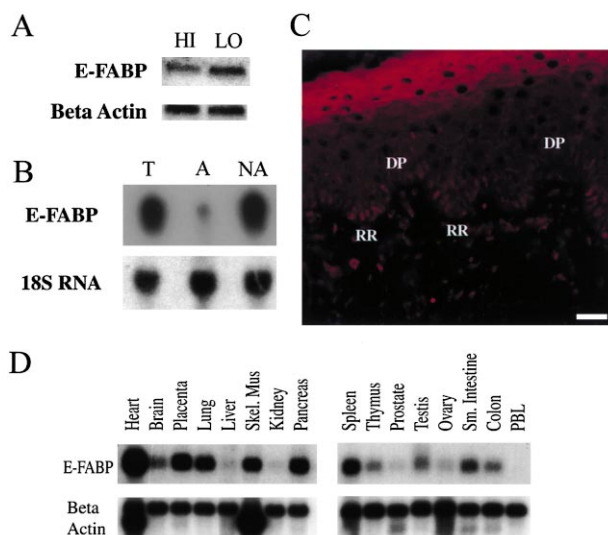


Fig. 3. Expression of PA-FABP in keratinocytes and other cell types. A,B,D: Northern blots of keratinocytes (A,B) or multiple tissues (D) were hybridised with a probe to PA-FABP and with β -actin (A,D) or 18S RNA (B) as a loading control. A: Keratinocytes were fractionated by FACS on the basis of high (HI) or low (LO) β 1 integrin expression. B: Unfractionated keratinocytes (total population; T) were compared with cells that adhered to type IV collagen within 20 min (adherent, stem cell enriched; A) and cells that did not adhere (enriched for transit amplifying and terminally differentiating keratinocytes; NA). C: Immunofluorescence staining of human epidermis with antibody to PA-FABP. DP, dermal papilla; RR, rete ridge. Scale bar: 50 μ m.

pendent pairs of cell lysates was analysed; a representative pair of gels is shown in Fig. 1 and the results of all the experiments are summarised in Table 1.

The overall pattern of expressed proteins was remarkably similar in each cell population (Fig. 1). The degree of contamination with suprabasal cells, evaluated by the level of involucrin expression [14], was low and equal in each fraction (Inv in Fig. 1). One unknown protein with a molecular mass of over 90 kDa was upregulated in the integrin bright population (SM in Fig. 1). Annexin II (Ann in Fig. 1) and psoriasis associated fatty acid binding protein (PA-FABP [8]; also known as epidermal FABP and FABP5 [16,17]) were upregulated in integrin dull cells, as were keratin related proteins of unknown origin (KV in Fig. 1). Although PA-FABP forms a complex with S100 proteins [16,17], the S100 proteins were not upregulated in the integrin dull fraction (see, for example, S100A7, also known as psoriasin, in Fig. 1).

3.2. Detection of differentially expressed genes by differential hybridisation of cDNA arrays

Filters arrayed with cDNAs of representative ESTs from the Unigene human gene catalogue were hybridised with labelled cDNA from either the stem cell enriched or the transit cell enriched population, sorted in exactly the same way as the metabolically labelled cells in the 2D gel electrophoresis experiments. The filters comprised two sets of approximately 16 000 known cDNAs spotted in duplicate. On average 6000 spots and their duplicates showed a detectable signal. Each pair of identical filter sets was hybridised with three pairs of independent probes from integrin bright and dull cells, so that each probe was hybridised at least once to each filter. Each

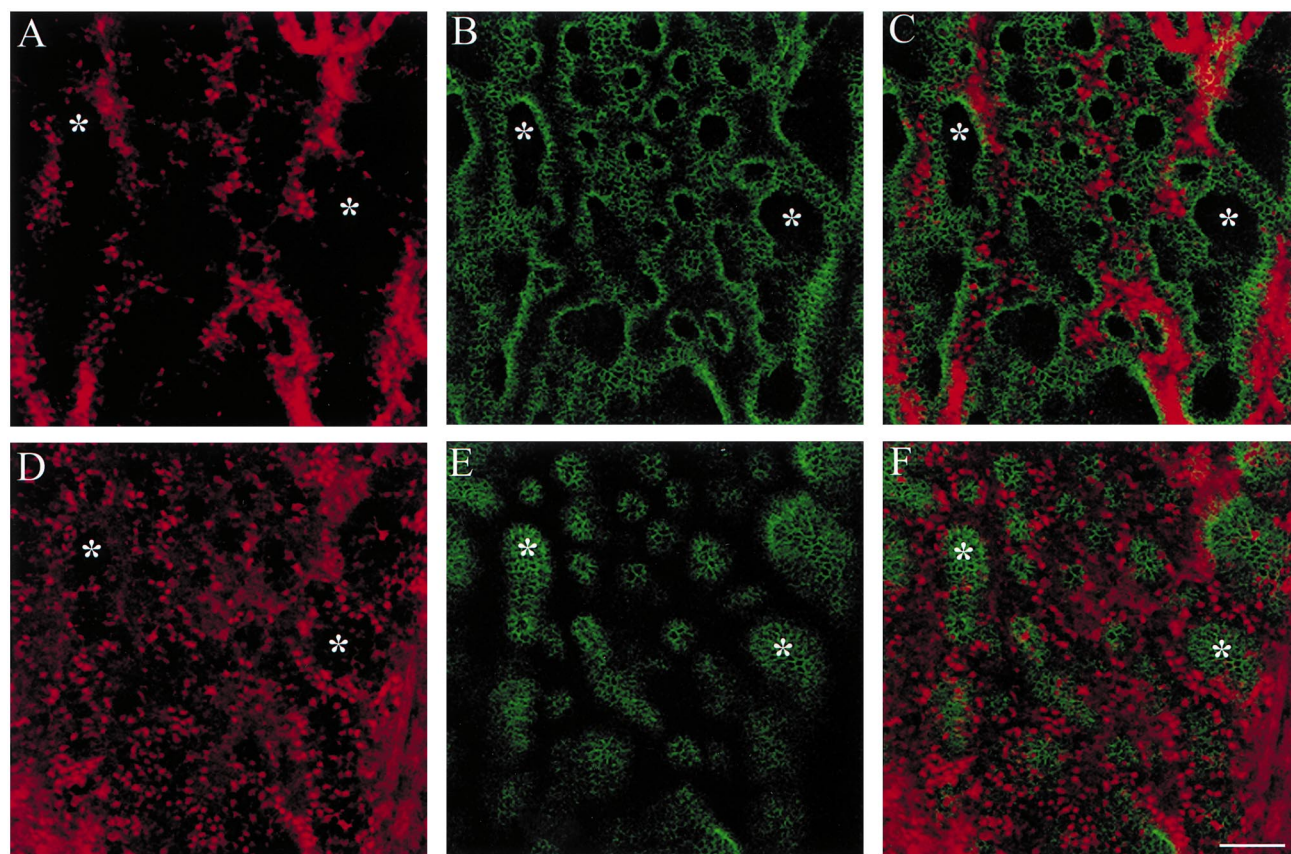


Fig. 4. Double immunofluorescence labelling of epidermal whole mount with antibodies to $\beta 1$ integrins (green) (B,C,E,F) and PA-FABP (red) (A,C,D,F). Two 0.5 μm optical sections (A–C and D–F) through the basal layer are shown. C and F are merged images of A and B and D and E, respectively. The asterisks show regions where the basal layer overlies a dermal papilla. Scale bar: 100 μm .

filter was also hybridised with M13 reverse primer to determine the amount of DNA on each spot. We were therefore able to exclude excessive variation in probe quality and to normalise for the DNA content of each spot on the filter pairs. The three resultant data sets were then normalised on the basis of total counts over all spots in the complex cDNA hybridisations, and evaluated in pairs and also as the sum of all equivalent experiments, to provide an average intensity at each spot for both the transit cell enriched and stem cell enriched populations.

The signal intensities of individual spots hybridised with RNA from integrin bright and dull cells were plotted against each other on a scatter plot, which automatically identifies duplicates and allows annotation (Fig. 2). Only cDNAs for which duplicate spots gave the same signal intensity were evaluated (see, for example, keratin 14 and S100A2 in Fig. 2A). Clones that appeared to be differentially expressed were

evaluated using the three normalised average signal intensities in the sum of the three experiments. None showed a difference in expression above 2-fold, the minimum considered significant. The PA-FABP signal was 2-fold more abundant in integrin bright than integrin dull cells (Fig. 2B), but since the absolute signal was relatively low, the result can only be taken as an indication of potentially higher expression in the transit amplifying population. No signal could be detected at the Annexin II spot (Fig. 2B).

3.3. Verification of differential expression of PA-FABP by Northern blotting

The upregulation of PA-FABP mRNA in transit amplifying cells was confirmed by Northern blotting of keratinocytes fractionated on the basis of $\beta 1$ integrin expression (Fig. 3A) or adhesion to type IV collagen (Fig. 3B). The PA-FABP signal was approximately 2-fold greater in integrin dull than

Table 1

Summary of the proteins that were differentially expressed in keratinocytes enriched for stem cells or transit amplifying cells

Protein	No. of gels in which protein observed	Upregulated in stem or transit?	No. of gels in which protein upregulated
PA-FABP	10	transit	10
Annexin II	8	transit	7
Stem marker	8	stem	5
Keratin related	10	transit	7

A total of 10 gel pairs representing 10 independent samples of FACS sorted keratinocytes were analysed.

integrin bright keratinocytes and showed a greater than 2-fold reduction in rapidly adhering cells (stem cell enriched) compared to cells that did not adhere to collagen within 20 min (enriched for transit amplifying and terminally differentiating cells) (Fig. 3B). When a probe to PA-FABP was hybridised to a Northern blot of a range of tissues, ubiquitous but variable expression was observed, except in peripheral blood leukocytes (PBL), in which no signal was detected (Fig. 3D).

3.4. Immunofluorescence staining of PA-FABP in human epidermis

In foreskin and breast skin, the $\beta 1$ integrin bright cells, which are stem cell enriched, overlie the tips of the dermal papillae, where the basal layer comes closest to the skin surface. The integrin dull cells occupy the rete ridges, where the basal layer projects into the dermis [3–5]. Cross-sections of neonatal foreskin stained with an antibody specific to PA-FABP showed staining in all the epidermal layers (Fig. 3C). Staining in basal keratinocytes was predominantly nuclear, whereas, as reported previously, in the upper layers cytoplasmic staining predominated [11,12,16]. Within the basal layer small groups of cells overlying the dermal papillae (DP in Fig. 3C) showed a reduction in PA-FABP staining compared to the cells of the rete ridges (RR in Fig. 3C).

The relative distribution of those basal cells expressing high levels of $\beta 1$ integrins or PA-FABP was examined by whole mount labelling of the epidermis in combination with confocal microscopy. A series of 0.5 μ m optical sections from a z-section through the basal layer of the epidermis is shown in Fig. 4. The positions of the dermal papillae are shown with an asterisk and correspond to clusters of cells with the highest levels of $\beta 1$ integrins (Fig. 4E,F). Nuclear staining of PA-FABP was weak in those cells (Fig. 4D,F). In contrast, strong nuclear staining of PA-FABP was observed in the integrin dull basal cells, corresponding to the rete ridges (Fig. 4A–C). The staining pattern with the antibodies to PA-FABP in both conventional sections and whole mounts of epidermis was thus consistent with PA-FABP being upregulated in transit amplifying cells.

4. Discussion

We used 2D protein gel electrophoresis and differential hybridisation of arrayed cDNA libraries to look for new markers of epidermal stem and transit amplifying cells. No genes with expression levels differing by more than a factor of two were detected in the hybridisation of the cDNA arrays. Several reported stem cell markers, such as $\beta 1$ integrins and Delta1, differ in expression relative to transit amplifying cells by approximately 2-fold [18] and so would not have been identified using the arrays, even if there was a corresponding 2-fold difference in RNA levels. No differential expression of several other proposed markers, such as keratin 15, E-cadherin and the $\alpha 6\beta 4$ integrin [18], was observed when analysed on the filters (data not shown).

Very few differences between the two subpopulations of keratinocytes were observed by 2D protein gel electrophoresis. Although over 2500 keratinocyte proteins can be identified on the gels [7], those that are optimally resolved are less than 90 kDa and can be labelled with methionine; thus many keratinocyte proteins, including the integrins, are not detectable. One potential stem cell marker was found, but it was not examined

further because it could not be identified from the existing database of keratinocyte proteins [7,8]. Upregulation of Annexin II, two keratin related proteins and PA-FABP was observed in the transit cell enriched fraction. The upregulation of PA-FABP was confirmed on the cDNA arrays and verified by Northern blotting of cultured keratinocytes and immunolocalisation in human epidermis.

PA-FABP is a small (15 kDa), conserved protein that is capable of binding long chain fatty acids, a characteristic of all members of the FABP family [19]. It is expressed in a wide range of cell types ([20,21]; Fig. 3D) and although usually regarded as a cytoplasmic protein, it does, like other family members, contain nuclear localisation sequences [22]. Expression of PA-FABP is upregulated in psoriatic epidermis, the tissue from which it was originally cloned [6]. It is interesting that PA-FABP was identified in our screen for transit amplifying cell markers, because psoriasis is proposed to involve an expansion of the transit amplifying compartment [23,24] and PA-FABP is upregulated by c-Myc [25], which is known to stimulate keratinocytes to become transit amplifying cells [26].

PA-FABP appears to be involved in the control of growth and differentiation in a number of cell types. The protein is upregulated during neuronal migration and differentiation [22,27] and during nerve repair after crushing injury [21]. Heart FABP, which is identical to mammary derived growth inhibitor, inhibits proliferation and stimulates differentiation of mammary epithelium [28]. Expression of intestinal FABP is tightly associated with the inhibition of DNA synthesis in the intestine [29]. It will be interesting to investigate whether PA-FABP regulates proliferation or differentiation of basal keratinocytes, whether this is dependent on its nuclear localisation, and how any effect is linked to fatty acid transport and metabolism [11,16].

Even allowing for the limitations of 2D protein gels and cDNA arrays, we must conclude that very few genes show significant differences in expression between basal keratinocytes fractionated by FACS with antibodies to $\beta 1$ integrins. It is possible that the degree of enrichment for stem and transit cells obtained on the basis of $\beta 1$ integrin expression [3,4] is too low to allow differentially expressed genes to be detected. It is also possible that differences in gene expression that are significant in intact epidermis are not maintained in culture: for example, in vivo a higher proportion of integrin dull than integrin bright cells are actively cycling [5], but this is not the case in vitro [3,4,26]. Another possibility is that the genes of interest are expressed at levels that are below the limits of detection of the methods we used. Finally, it could be that there really are very few intrinsic markers that distinguish stem from transit amplifying cells and that the most significant changes in gene expression are transient, as in the induction of c-Myc that can trigger exit from the stem cell compartment [26]. As we continue to explore the properties of epidermal stem cells we will need to design strategies to account for all these possibilities.

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