Evidence for filaggrin as a component of the cell envelope of the newborn rat

Susan RICHARDS,* Ian R. SCOTT,† Clive R. HARDING,† J. Eryl LIDDELL,* Gillian M. POWELL* and C. Gerald CURTIS*

*Department of Biochemistry, University College, P.O. Box 78, Cardiff, Wales, U.K., and †Unilever Research, Colworth House, Sharnbrook, Bedford, U.K.

A substrate of transglutaminase, specific to the epidermis, was identified, by fluorescent and radioactive labelling with the lysine analogues dansylcadaverine and [¹⁴C]putrescine respectively, in newborn-rat epidermal homogenates and whole-skin organ cultures. The labelled analogues were preferentially incorporated into the stratum-corneum protein filaggrin in a Ca²⁺-dependent manner in both 'in vitro' systems. When filaggrin was labelled *in vivo* with [³H]histidine and then incubated with rat epidermal preparations, the label was rendered SDS/thiol-insoluble. Incorporation of [³H]filaggrin into the insoluble envelope fraction was Ca²⁺-dependent and inhibited by EDTA and exogenous amines. Antisera to newborn-rat filaggrin cross-reacted with purified newborn-rat cell envelopes, and this reaction was blocked by adsorbing the antiserum with purified filaggrin. Quantification of the 'envelope-bound' filaggrin showed it to be a significant component, accounting for approx. 10% of the cell-envelope protein.

INTRODUCTION

Filaggrin is a major cellular protein produced during the latter stages of epidermal differentiation. It is synthesized in the granular layer in the form of a high-molecular-mass (> 300 kDa in the rat) heavily phosphorylated precursor, profilaggrin, which accumulates in the keratohyalin granules. During the transition from the granular layer to the stratum corneum the profilaggrin is dephosphorylated and proteolytically cleaved to produce (in the rat) one protein of molecular mass 45 kDa, namely filaggrin.

There are currently two proposed functions for filaggrin: (1) on the basis of its ability to aggregate keratin filaments *in vitro* it has been suggested that it is responsible for the close-packed keratin pattern seen in the stratum corneum (Dale *et al.*, 1978; Steinert *et al.*, 1981). However, more recently it has been proposed (2) that the primary function of filaggrin is to generate a concentrated pool of free amino acids within the stratum corneum (Scott *et al.*, 1982), hence greatly adding to the moisture-binding capacity of the stratum corneum (Scott, 1981; Barrett & Scott, 1983).

We report here that filaggrin is a substrate for the enzyme epidermal transglutaminase *in vitro* and is a putative component of the cross-linked epidermal-cell envelope *in vivo*.

EXPERIMENTAL

Materials

Newborn (2–3-day-old) rats of the Colworth–Wistar or MRC-hooded strain were used throughout. [1,4-¹⁴C]-Putrescine dihydrochloride of specific radioactivity 118 mCi/mmol was supplied by Amersham International. It was evaporated to dryness under N₂ and redissolved in unlabelled-putrescine solution (10 mM in

50 mM-Tris/HCl, pH 7.5) to give a final specific radioactivity of 5 mCi/mmol which was used in all experiments. L-[2,5-³H]Histidine was also obtained from Amersham International as a solution of specific radioactivity 1 mCi/ml in 2% (v/v) ethanol, and it was used for injections as supplied. Monodansylcadaverine and phenylmethanesulphonyl fluoride (PMSF) were supplied by Sigma. Dithiothreitol (DTT), and Dispase (grade II) were obtained from Boehringer. Culture media was supplied by Flow Laboratories. Materials for liquid-scintillation counting were supplied by May and Baker, with the exception of Soluene 350, which was obtained from Packard. Electrophoresis chemicals were all of the highest available grade and were supplied by BDH. Unless stated otherwise, all other chemicals were of analytical-reagent grade.

Methods

Synthesis of dansylcadaverine fumarate. Dansylcadaverine was converted into the fumarate salt as described by Stenberg *et al.* (1975) in order to increase its solubility, and it was used in this form in all experiments.

Incorporation of synthetic amines into proteins of epidermal homogenates. Newborn (2–3-day-old) rats were killed by cervical dislocation. Epidermis was isolated either by heat separation (immersion in a water bath at 60 °C for 1 min) or by freeze-scraping (Scott, 1981) and homogenized at 4 °C (1 epidermis/ml) in Tris/EDTA buffer, pH 7.5, containing Tris/HCl (50 mM), EDTA (1 mM) and PMSF (2 mM), by using a Kontes Dual 22 tissue grinder. Incubation mixtures comprised epidermal homogenate (500 µl), dansylcadaverine (1 mM) or [¹⁴C]-putrescine (1 mM), and CaCl₂ (5 mM) or EDTA (10 mM). Incubation mixtures were adjusted to a final volume (1 ml) with the Tris buffer and were incubated at 37 °C for

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (for composition and pH, see the text); FITC, fluorescein isothiocyanate; e.l.i.s.a., enzyme-linked immunosorbent assay.

2 h, after which time they were prepared for SDS/polyacrylamide-gel electrophoresis (SDS/PAGE).

Incorporation of dansylcadaverine into epidermal proteins in whole-skin organ cultures. Pieces of full-thickness newborn-rat skin (1 cm × 1 cm), were floated, dermis-side-down, on standard Dulbecco's modified Eagle medium (0.5 ml) containing dansylcadaverine (1 mM), at 37 °C in a humidified CO₂/air (19:1) atmosphere for 1 h. Epidermis was then isolated by freeze-scraping and homogenized in SDS lysis buffer, pH 8.0, comprising Tris/HCl (100 mM), glycerol (10%), SDS (2%), EDTA (1 mM) and PMSF (2 mM) before electrophoresis.

The viability of this culture system was assessed by its ability to incorporate [³H]histidine (100 μl; 100 μCi) into profilaggrin as described by Harding & Scott (1983). In addition, pieces of cultured skin that had been incubated in the presence of dansylcadaverine were fixed, embedded, stained with haematoxylin and eosin, and sectioned (8 μm thickness) for histological examination.

SDS/PAGE. Samples (except those from organ-culture experiments) were adjusted to give the following final concentrations: SDS, 2% (w/v); EDTA, 1 mM; PMSF, 2 mM; glycerol, 10% (v/v). All samples were made 20 mM with respect to DTT, incubated at 60 °C for 30 min, and centrifuged (10 000 *g* for 5 min). Bromophenol Blue tracking dye was added to the supernatant, and aliquots (25 μl) were analysed on mini gradient slab gels as described by Harding & Scott (1983).

Radiolabelled proteins were quantified by slicing the gels transversely into 1 mm pieces, which were solubilized overnight at 50 °C in Soluene 350/water/PPO (2,5-diphenyloxazole)/toluene (20:2:1:177, v/v/w/v), then assayed for radioactivity by liquid-scintillation counting. Alternatively, radiolabelled proteins were identified by fluorography on Fuji R-X medical X-ray film as described by Laskey & Mills (1975).

Preparation of [³H]filaggrin. Newborn (1-day-old) rats were each injected subcutaneously with [³H]histidine (20 μl, 20 μCi) by using a Hamilton PB600 repeating dispenser. Animals were killed 24 h later and the epidermis was isolated by freeze-scraping, homogenized (1 epidermis/ml) in a urea buffer comprising urea (8 M), Tris/HCl (10 mM), EDTA (1 mM) and PMSF (2 mM), pH 8.0, and incubated at 37 °C for 30 min to extract the urea-soluble proteins.

The extract was passed through a small column (1.5 cm × 10 cm) of DE52 DEAE-cellulose in the same buffer to remove keratin, nucleic acids and profilaggrin. The resulting partially purified filaggrin was stored at -70 °C in the urea buffer and desalted immediately before use into 10 mM-Tris/HCl buffer, pH 7.5, by using a centrifugal desalting method (Helmerholst & Stokes, 1980).

The purity of the prepared filaggrin was determined by PAGE followed by gel slicing. The protein content of filaggrin preparations was assayed by the ninhydrin method (Moore & Stein, 1948) after complete acid hydrolysis in 6 M-HCl under N₂ at 110 °C for 18 h, glutamic acid being used as standard.

Incorporation of [³H]filaggrin into the SDS/thiol-insoluble fraction of newborn-rat epidermal homogenates. Epidermis was isolated by freeze-scraping, homogenized

in Tris/EDTA buffer, pH 7.5, and aliquots (225 μl) were incubated with [³H]filaggrin (10.6 × 10⁴ μCi, 65 μg) and either CaCl₂ (5 mM) or EDTA (10 mM). Samples were incubated at 37 °C for 3 h, after which time a portion of the reaction mixture (250 μl) was removed and the reaction stopped by the addition of SDS and DTT to final concentrations of 2% and 20 mM respectively. The remainder of the reaction mixture was incubated for a further 3 h, after which the reaction was terminated in an identical manner.

After the addition of SDS/DTT, all samples were prepared for SDS/PAGE and electrophoresed as described above.

The pellet was washed exhaustively with buffer containing SDS/DTT, before being solubilized in Luma-solve tissue solubilizer (200 μl), neutralized, and counted for radioactivity in Lumagel scintillant.

Preparation of cell envelopes. Newborn rats were killed, and the whole dorsal and ventral skin was removed and floated dermis-side-down for 90 min at 37 °C on a solution of phosphate-buffered saline (PBS; 0.8% NaCl/0.02% KCl/0.02% KH₂PO₄/0.115% NaH₂PO₄, pH 7.4 containing Dispase (0.25%). The epidermis was then peeled away from the dermis.

The epidermis was rinsed in SDS lysis buffer and homogenized in the same buffer. The homogenate was incubated at 60 °C for 30 min, then centrifuged (10 000 *g* for 5 min), and the pellet was washed exhaustively with the same buffer. The pellet was freeze-dried, and the SDS was removed by ion-pair extraction (Henderson *et al.*, 1979). Protein was determined by dry weight.

Preparation of antisera. The preparation and purification of rabbit anti-(rat filaggrin) antiserum was described previously (Scott & Harding, 1986).

Immunofluorescence microscopy. Newborn-rat skin was fixed for 1 h in 3.5% freshly hydrolysed paraformaldehyde in PBS, then washed overnight in 15% (w/v) sucrose in PBS. The tissue was embedded in Tissue-Tek OCT compound, snap-frozen in isopentane/liquid N₂, and sectioned at 8 μm thickness. Sections were blocked with normal goat serum (50%, v/v, in PBS), for 15 min at room temperature, allowed to react with anti-filaggrin antiserum (usually 1:50 dilution) for 30 min at room temperature, and washed in PBS (30 min at room temperature). Second antibody [affinity-purified fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG] at a dilution of 1:50 was then applied and incubated at room temperature for 30 min; sections were then washed as described above, mounted in glycerol/*p*-phenylenediamine medium (Johnson & de C. Nogueira-Arainjo, 1981) and examined for fluorescence.

Controls with non-immune serum or the fraction of immune serum that did not bind to a filaggrin-affinity column gave diffuse fluorescence throughout the epidermis and stratum corneum. Either affinity-purified non-immune serum or FITC goat anti-(rabbit IgG) alone gave similar results, with only faint background staining.

Localization of filaggrin in purified cell envelopes using a non-competitive e.l.i.s.a. technique. Cell envelopes were resuspended (1 mg/ml) in Na₂CO₃/NaHCO₃ coating buffer (15 mM, pH 9.6) containing Thimerosal (0.02%; Sigma).

The e.l.i.s.a. protocol was as follows. Cell envelopes (100 μ l) were adsorbed on to 96-well microtitre plates (Falcon 3915; Becton-Dickinson Labware, Oxnard, CA, U.S.A.), at 4 °C overnight. Plates were washed three times with PBS containing Tween-20 (0.05%, Bio-Rad) and blocked with bovine serum albumin (1% in PBS, 100 μ l/well) for 1 h at room temperature. After washing (as described above), affinity-purified rabbit anti-(rat filaggrin) antiserum (100 μ l) diluted in PBS was added to each well and incubated for 1 h at room temperature. The washing procedure was repeated, and anti-rabbit IgG-alkaline-phosphatase conjugate (Sigma), diluted 1:1000 in PBS (100 μ l) was added to each well and incubated at room temperature for 1 h. After further washing in the PBS/Tween mixture, followed by one wash in distilled water, aliquots (100 μ l) of disodium *p*-nitrophenyl phosphate (1 mg/ml) in glycine buffer (0.1 M, pH 10.4), containing $MgCl_2$ (1 mM) and $ZnCl_2$ (1 mM), were added. After 30 min the reaction was terminated by the addition of 1 M-NaOH (30 μ l/well) and the A_{405} measured by using an enzyme-immunoassay reader (Bio-Rad).

Inhibition of anti-filaggrin antibody binding to cell envelopes after adsorption of the antibody with purified filaggrin. Plates were coated with cell envelopes (100 μ g/100 μ l) overnight at 4 °C. Anti-filaggrin antibody (1:4000 in PBS) was pre-incubated for several hours at 4 °C with various concentrations (2–8 μ g/100 μ l) of purified filaggrin, and this mixture was applied to the coated plates instead of the antibody alone. The remainder of the protocol was carried out as described above.

RESULTS

Ca^{2+} -dependent incorporation of primary amines into homogenates and organ cultures of rat epidermis

SDS/PAGE of rat epidermal homogenates which had been incubated with either dansylcadaverine or [^{14}C]-putrescine showed two major protein bands which had been labelled with the fluorescent dansyl group or ^{14}C (Figs. 1 and 2). One band was of very high molecular mass and did not penetrate, to any significant extent, the 3% stacking gel. The second labelled band was at approx. 45000 M_r and corresponded in mobility with filaggrin. The identity of this protein was confirmed by two-dimensional electrophoresis. Labelling of filaggrin was Ca^{2+} -dependent and was prevented by pre-incubation of homogenates with iodoacetamide. Incorporation of dansylcadaverine into filaggrin was also observed after whole-skin organ culture in media containing dansylcadaverine (Fig. 3). The viability of this culture system was assessed by studying the incorporation of [3H]-histidine into the filaggrin precursor profilaggrin (Fig. 4). The label was incorporated into profilaggrin in a manner identical with that previously demonstrated *in vivo* (Scott & Harding, 1981), indicating that the skin was viable under these conditions. In addition, a histological comparison of skin sections incubated in the presence or absence of dansylcadaverine showed no visible structural differences.

Ca^{2+} -dependent incorporation of [3H]filaggrin into cell envelopes

When purified [3H]histidine-labelled filaggrin (Fig. 5) was incubated with epidermal homogenate, the radiolabel

Fig. 1. Incorporation of dansylcadaverine into proteins of epidermal homogenates

Aliquots (30 μ l) of the SDS/DTT-soluble epidermal extract that had been incubated with dansylcadaverine (1 mM) as described in the text were analysed by SDS/PAGE. (a) Shows the fluorescence profile after illumination with u.v. light. Track 1 shows the profile in the presence of $CaCl_2$ (5 mM), and the filaggrin band is labelled 'F'; there is also a very high-molecular-mass fluorescent band at the top of the stacking gel indicated by an arrow ($\blacktriangleright$). Track 2 shows the profile in the presence of EDTA (10 mM). The intense band of fluorescence at the dye front is free dansylcadaverine. (b) Shows the protein profiles of the same samples after staining with Kenacid Blue dye. The filaggrin band is labelled 'F'. Tracks 3 and 4 show the protein profile in the absence of dansylcadaverine but in the presence of $CaCl_2$ (5 mM).

was incorporated into an SDS/thiol-insoluble fraction (cell envelope) in three identical experiments. The Ca^{2+} -dependent incorporation was linear over 6 h and was inhibited by EDTA and exogenous amines (Fig. 6). SDS/PAGE of the SDS/thiol-soluble extract showed that the remaining 3H was still associated with the filaggrin band, and in experiments in which dansylcadav-

Fig. 2. Incorporation of [^{14}C]putrescine into proteins of epidermal homogenates

Aliquots (30 μl) of the SDS/DTT-soluble epidermal extract that had been incubated in the presence of [^{14}C]putrescine (1 mM, 5 mCi/mmol) as described in the text were analysed by SDS/PAGE. Stained gels were sliced and assayed for radioactivity by liquid-scintillation counting. (a) Shows the profile of radioactivity in the presence of CaCl_2 (5 mM), whereas (b) shows the profile in the presence of EDTA (10 mM). The peak of radioactivity of 45000 M_r ('45K') is assumed to be filaggrin. Under these conditions there is no incorporation into the filaggrin precursor profilaggrin [M_r approx. 300000 ('300K')].

erine inhibited the incorporation of [^3H]filaggrin into the cell envelope, the filaggrin band was also fluorescently labelled.

Localization of filaggrin in newborn-rat cell envelope by an e.l.i.s.a.

When purified cell envelopes were used as the antigen in the e.l.i.s.a. procedure, there was measurable reactivity between the antigen and the anti-filaggrin antibody. The antigen and antibody dilution curves (Fig. 7) show a linear relationship between the antigen (cell envelopes) and the antibody (anti-filaggrin). Moreover, the relationship was identical with that observed when purified filaggrin was used as the antigen.

The specificity of the response was confirmed by the inhibition study in which anti-filaggrin was adsorbed with purified filaggrin (at increasing concentrations) to block the available filaggrin-binding sites on the antibody before being applied to plates coated with cell envelopes. The inhibition observed was dose-dependent and was similar to that observed with purified filaggrin as the antigen (Fig. 8), thus confirming the specificity of the reaction and ruling out the possibility that the binding was due to some contaminant in the antiserum.

Fig. 3. Incorporation of dansylcadaverine into epidermal proteins in whole-skin organ culture

Whole-skin organ-culture experiments were carried out in the presence of dansylcadaverine (1 mM) as described in the text. Aliquots (30 μl) of the SDS/DTT-soluble supernatant were analysed by SDS/PAGE. (a) Shows the fluorescence profile under u.v. light of proteins labelled with dansylcadaverine. Tracks 1 and 2 show the profile in the presence of CaCl_2 (5 mM), whereas tracks 3 and 4 show the profile in the presence of EDTA (10 mM). The filaggrin band is denoted by 'F'. The intense band of fluorescence at the dye front (labelled d_f) is due to free dansylcadaverine diffusing back into the gel during photography. (b) Shows the protein profile of the same samples after staining with Kenacid Blue dye. The filaggrin band is labelled 'F' and has an M_r of 45000 ('45K'). The band at approx. 300000 M_r ('300K') is profilaggrin.

Quantification of filaggrin in cell envelopes

Fig. 7 shows the linear relationship between envelope concentration and immunoreactivity, showing that envelopes contain a measurable amount of filaggrin. However, in order to quantify the amount of filaggrin in envelopes, it was necessary to determine (a) the actual amount of filaggrin bound to the e.l.i.s.a. plate when setting up the standard calibration curve and (b) the actual amount of cell-envelope protein bound.

The relationship between the amount of filaggrin

Fig. 4. Incorporation of radioactivity into profilaggrin after whole-skin organ culture in the presence of $[^3\text{H}]$ histidine and dansylcadaverine

Whole newborn-rat skin was incubated in medium containing $[^3\text{H}]$ histidine ($100\ \mu\text{l}$, $100\ \mu\text{Ci}$), and the epidermis was isolated by freeze-scraping and homogenized as described in the text. Aliquots ($30\ \mu\text{l}$) of the SDS/DTT-soluble material were analysed by SDS/PAGE and assayed for radioactivity after gel slicing. The highly labelled band ('PF') has an M_r in excess of 300 000 and is profilaggrin. The band labelled 'F' is filaggrin.

Fig. 5. Purification of $[^3\text{H}]$ filaggrin

A profile of radiolabelled filaggrin after purification on DEAE-cellulose DE52, analysis by SDS/PAGE and gel slicing is shown. The filaggrin band is labelled 'F' [M_r 45 000 ('45K')].

Fig. 6. Incorporation of $[^3\text{H}]$ filaggrin into the cell-envelope fraction *in vitro*

The incorporation of $[^3\text{H}]$ filaggrin into the SDS/DTT-insoluble fraction of newborn-rat epidermal homogenate after incubation over 6 h in the presence of CaCl_2 (5 mM; ●), CaCl_2 (5 mM) and dansylcadaverine (1 mM) (○) and EDTA (10 mM; ▲) is shown.

bound to the plate and the immunoreactivity was determined by using $[^3\text{H}]$ histidine-labelled filaggrin. E.l.i.s.a. plates were coated with ^3H -labelled filaggrin ($100\ \mu\text{l}$: 1.79×10^4 d.p.m./ μg) at concentrations ranging from 10 to 25 $\mu\text{g}/\text{well}$ at 4°C overnight in HCO_3^- coating buffer. The plates were washed with PBS/Tween to remove the unbound $[^3\text{H}]$ filaggrin, and aliquots ($300\ \mu\text{l}$) of 2 M-HCl were applied to each well to remove the bound filaggrin. The plate was incubated at 37°C for 60 min and the HCl was then transferred into a scintillation vial. The wells were washed with $2 \times 250\ \mu\text{l}$ aliquots of the acid and the total, 700 μl , was neutralized with 2 M-NaOH and assayed for radioactivity by liquid-scintillation counting. On the same plates, duplicate samples were assayed by using the e.l.i.s.a. protocol outlined above, and the results showed a linear relationship between the measurable alkaline phosphatase activity (i.e. bound anti-filaggrin) and the amount of $[^3\text{H}]$ filaggrin adsorbed to the wells.

Binding of epidermal-cell envelopes was quantified by removing the bound protein from the washed plate by incubation with HCl as described above. The HCl was removed by freeze-drying, and samples were either (a) redissolved in 6 M Aristar-grade HCl (500 μl), sealed under vacuum, hydrolysed at 110°C for 18 h and assayed for protein content by using the ninhydrin method with glutamic acid as standard, or (b) samples were redissolved in 0.1 M-NaOH (500 μl) and protein was determined by using a modified Lowry method, using bovine serum albumin as standard. Wells which had been coated with HCO_3^- coating buffer only were processed in an identical manner and served as blanks in each assay.

The binding capacity of the e.l.i.s.a. plates varied from one batch to another, and it was therefore essential that measurements of filaggrin binding, envelope binding and the measurements of filaggrin in cell envelopes (using the e.l.i.s.a. assay) were carried out on the same plates. In

Fig. 7. Reactivity of rabbit anti-(rat filaggrin Ig) with filaggrin or cell envelopes

(a) Shows the specific antibody binding to purified cell envelopes (●) or pure filaggrin (○) adsorbed to polystyrene e.i.s.a. plates at concentrations between 6.25 and 100 $\mu\text{g}/\text{well}$. In each case immunopurified anti-rat filaggrin Ig at a dilution of 1:100 in PBS was used. (b) Shows the effect of antibody dilution (in PBS) after the adsorption of purified cell envelopes (●; 100 $\mu\text{g}/\text{well}$) or pure filaggrin (○; 50 $\mu\text{g}/\text{well}$) to e.i.s.a. plates. Each value was corrected for the level of binding observed in the presence of non-immune serum.

Fig. 8. Inhibition of antibody binding to immobilized filaggrin or cell envelopes after preincubation of antibody with soluble filaggrin

Anti-filaggrin at 1:1000 dilution was incubated with soluble filaggrin (0–8 μg). These preparations were then applied to e.i.s.a. plates precoated with either filaggrin (12.5 $\mu\text{g}/\text{well}$; □) or cell envelopes (100 $\mu\text{g}/\text{well}$; ■). Antibody binding to the solid phase was determined as described in the text. Each value was corrected for the level of binding observed in the presence of non-immune serum.

this way the filaggrin concentration could be expressed as '% filaggrin in the bound envelope fraction' (Table 1).

The results summarized in Table 1 demonstrate that filaggrin comprises approx. 10% of the epidermal-cell envelope of the newborn rat.

Localization of filaggrin in newborn-rat stratum corneum by immunofluorescence microscopy

After immunofluorescent labelling of newborn-rat epidermis, the cell envelopes of the upper stratum corneum were found to cross-react with antiserum to filaggrin (Fig. 9), indicating that measurable amounts of filaggrin were localized in this region.

Table 1. E.i.s.a. assays of the percentage of filaggrin in purified cell envelopes from newborn rat skin

The mean values calculated from the data were 9.4 ± 2.9 [Lowry method (L), $n = 9$] and 16.17 ± 4.13 [Ninhydrin method (N), $n = 9$]. Abbreviation used: n.d., not determined.

Expt. no.	Envelopes applied/well (μg) ... Protein assay ...	Filaggrin in the bound envelopes (%)					
		100		75		50	
		I	N	L	N	L	N
1		5.7	14.3	n.d.	n.d.	6.8	27.2
		6.9	11.5	n.d.	n.d.	12.4	17.0
2		11.4	6.0	n.d.	n.d.	n.d.	n.d.
		10.2	8.0	n.d.	n.d.	n.d.	n.d.
3		11.5	12.1	n.d.	n.d.	n.d.	n.d.
		n.d.	n.d.	6.5	10.7	n.d.	n.d.
		n.d.	n.d.	13.0	9.2	n.d.	n.d.

Fig. 9. Immunofluorescence localization of filaggrin

(a) Indirect immunofluorescence of newborn-rat epidermis using antiserum to filaggrin. (b) Phase-contrast micrograph (a mirror image of the same section shown in a).

DISCUSSION

Previous studies designed to identify substrates of epidermal transglutaminase(s) in newborn-rat skin demonstrated that dansylcadaverine was incorporated in a Ca^{2+} -dependent manner into an SDS-soluble protein of M_r approx. 50000 (Hanigan & Goldsmith, 1978). However, the identity of this protein was unknown, and no further characterization studies were carried out.

From the data described here in which the synthetic amines dansylcadaverine and putrescine are incorporated predominantly into the major stratum-corneum protein filaggrin (M_r 45000), it seems possible that the 50000- M_r protein of Hanigan & Goldsmith (1978) is filaggrin.

Unfortunately this method of identifying endogenous substrates for transglutaminases in homogenates and other cell fractions can be misleading, presumably because of the changes in topography of enzymes, Ca^{2+} and/or protein substrates. However, in the present study filaggrin was also a substrate for amine incorporation in whole-skin organ cultures in which the viability and structural integrity was assessed by [^3H]histidine incorporation into profilaggrin and by optical microscopy respectively. Thus filaggrin could be a transglutaminase substrate *in vivo* and become cross-linked into the insoluble epidermal-cell envelope, which is a characteristic feature of fully differentiated keratinocytes (Sun & Green, 1976; Matoltsy, 1977; Rice & Green, 1979).

When this possibility was explored with immunological techniques, not only was filaggrin present in the purified insoluble cell envelope, but it was present in significant amounts accounting for approx. 10% of the envelope protein.

Previous work (Scott *et al.*, 1982) had demonstrated that, after the intradermal injection of a pulse dose of [^3H]histidine into rat skin, the label first appeared in profilaggrin, then filaggrin, followed by degradation to

free histidine and its derivative, urocanic acid (Scott, 1981). However, not all the ^3H appears as free histidine/urocanic acid. A proportion (approx. 20%) is not degraded, but is rendered insoluble in SDS and reducing agents (Scott *et al.*, 1982), and it was suggested that transglutaminase-mediated cross-linking into the cell envelope had occurred. However, there was no evidence at the time that this material was derived from [^3H]filaggrin.

In retrospect this role for filaggrin as a glutamine donor protein for epidermal transglutaminase (and hence as an envelope component) is not altogether unexpected, because filaggrin is composed of approx. 20% glutamic acid/glutamine residues (Dale, 1977; Richards, 1987) and is therefore ideally suited as an amine acceptor. Filaggrin shares this high proportion of glutamine residues with the other previously isolated envelope precursors, namely involucrin (Rice & Green, 1979; Watt & Green, 1981) and keratolinin (Buxman *et al.*, 1976, 1980). It is also interesting that involucrin is not to be found in the rodent species, being associated solely with man and higher primates (Rice & Thacher, 1986). The ratio of filaggrin to keratin is much lower in human epidermis when compared with rodents (cf. Figs. 2 and 4 of Harding & Scott, 1983), which would seem to indicate that these two proteins have evolved to serve a similar role in their respective species.

In conclusion, filaggrin has been identified as a novel glutamine-donor substrate of epidermal transglutaminase in the newborn rat and as a component of the epidermal-cell envelope *in vivo*.

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