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The Occurrence of Profilaggrin and Its Processing in Cultured Keratinocytes

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An affinity-purified antibody to rat filaggrin detects filaggrin and profilaggrin in extracts of newborn rat epidermis, and a monoclonal antibody to human filaggrin, HF-1, detects the two proteins in extracts of human epidermis. Immunohistologic studies show that HF-1 reacts with keratohyaline granules of human epidermis and those seen in cultured human keratinocytes. Immunoblotting studies have demonstrated that profilaggrin is synthesized in both cultured human keratinocytes and in a long-lived line of cultured rat keratinocytes, but only in the latter is the protein processed to a product of the molecular weight of filaggrin.

The epidermis of a number of mammalian species including humans has been shown to contain "histidine-rich" basic proteins [1–4] and the first of these to be purified to homogeneity was isolated from newborn rat stratum corneum. Rat stratum corneum basic protein was found to have a M_r of about 50,000 [5] and an amino acid composition similar to material derived from keratohyaline granules [6]. This protein was shown to promote the in vitro aggregation of keratin filaments [7] and was then given the functional name, filaggrin (filament aggregating protein) [8]. The aggregated filaments showed a picture of light-staining filaments surrounded by a dark matrix when viewed by transmission electron microscopy. This was very similar to the keratin pattern previously observed in human stratum corneum by electron microscopy which had been postulated to result from an interaction of tonofilaments and keratohyaline protein [9].

Filaggrin was found to be derived from a phosphorylated basic protein of similar M_r but more recent studies have shown that both are the degradation products of a large phosphorylated precursor protein of M_r greater than 200,000 [10,11]. This protein, named profilaggrin, was very sensitive to proteolysis, and a number of its breakdown products were present in epidermal extracts [10]. The metabolism of profilaggrin to filaggrin appears to occur in steps involving both dephosphorylation and proteolysis, but the mechanism of this conversion has not been precisely defined.

This process as well as the further metabolism of filaggrin [12,13] might be better studied in an in vitro system where conditions can be more rigorously controlled. Recently, we described the presence of filaggrin and filaggrin-related proteins in a line of long-lived keratinocytes [14]. This paper describes further immunologic studies of filaggrin-related proteins in rat and human cultured keratinocytes using an affinity-purified antibody to rat filaggrin and a monoclonal antibody to human filaggrin. These observations provide the basis for investigating the factors that regulate filaggrin formation and degradation.

MATERIALS AND METHODS

Culture Techniques

The derivation of the newborn rat keratinocyte line was described in a previous paper [14]. For the experiments described here, 18th passage keratinocytes which had remained frozen in 10% glycerol for over 2 years were thawed and recultured. These cells were grown in flasks in Eagle's minimum essential medium containing 10% fetal calf serum (FCS) in a 5% CO_2 atmosphere at 37°C. No 3T3 or other feeder cells were used in experiments with rat keratinocytes.

Human keratinocytes were grown as described earlier [15]. Infant human foreskins were obtained within 2 h of circumcision, rinsed extensively in phosphate-buffered saline (PBS) containing 100 U penicillin and 100 μg streptomycin, and then cut into 0.5 × 2 cm strips and placed in 0.25% trypsin overnight at 4°C. The next day the strips were removed from the trypsin, the epidermis separated with forceps, and the dermis and other tissue discarded. Separated epidermis was transferred to Dulbecco's modified Eagle's medium (DMEM) containing 20% FCS, 0.4 μg/ml hydrocortisone, 10^{-9} M cholera toxin, 50 U/ml penicillin, and 50 μg/ml streptomycin and agitated vigorously by pipetting. The medium containing the suspended cells was decanted away from the undisaggregated epidermis and centrifuged. The cell pellet was resuspended in the above medium and the cell density determined by counting electronically. Primary cultures were initiated in 35-mm petri dishes using 140,000 mitomycin C-treated 3T3 cells and 200,000 keratinocytes per dish. Medium was replaced 2 or 3 times per week and at the first change epidermal growth factor (EGF) at 10 ng/ml was added. Cultures were confluent at 2 weeks or less, and at 10–14 days after initiation were treated with 0.02% EDTA to remove fibroblasts. Keratinocytes were removed in 0.05% EDTA and 0.05% trypsin, centrifuged, and resuspended in medium. Subcultures were initiated as described for primary cultures and were passaged once per week. The current studies were done on subcultured cells between their 2nd and 10th subculture.

Electrophoretic and Immunologic Methods

Electrophoresis was done according to Laemmli [16] for one-dimensional gels and according to O'Farrell et al [17] for two-dimensional gels. Immunoblotting was done according to Towbin et al [18]. Polypeptides separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were electrophoretically transferred to nitrocellulose using the Bio-Rad transblot apparatus. Strips containing rat proteins were blocked with 3% gelatin in 0.5 M NaCl and 20 mM Tris/HCl, pH 7.5, (TBS) for 1 h and incubated in a 1:2000 dilution of an affinity-purified rabbit antibody to rat filaggrin. They were washed with 2 changes of TBS containing .05% Tween 20 and incubated in a 1:200 dilution of an affinity-purified, peroxidase-conjugated goat antibody to rabbit IgG (Bio-Rad) in TBS containing 1% gelatin for 1 h. The strips were washed twice in TBS containing .05% Tween 20 and finally developed with the Bio-Rad color development reagent.

Strips containing human proteins were blocked with 3% bovine

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Abbreviations:
- DMEM: Dulbecco's modified Eagle's medium
- EGF: epidermal growth factor
- FCS: fetal calf serum
- HAT: hypoxanthine, aminopterin, thymidine
- HT: hypoxanthine, thymidine
- PAGE: polyacrylamide gel electrophoresis
- PBS: phosphate-buffered saline
- PMSF: phenylmethylsulfonylfluoride
- SDS: sodium dodecyl sulfate
- TBS: 0.5 M NaCl and 20 mM Tris/HCl, pH 7.5

serum albumin in PBS for 3 h, rinsed with PBS, and reacted with the hybridoma conditioned medium which contained the monoclonal antibody to human filaggrin (HF-1). After rinsing, the strips were reacted with an fluorescein isothiocyanate-conjugated goat antibody to mouse IgG diluted 1:40 in PBS for 1 h. Strips were visualized and photographed using long-wave ultraviolet irradiation.

Preparation of Filaggrins

Purification of human filaggrin was accomplished similarly to the method of Lynley and Dale [4]. Whole-thickness human skin was obtained from amputated legs with a Castroveijo keratotome. The skin slices were soaked in .25 M NH_4Cl, pH 9.5, for 20 min at 4°C and separated from the dermis with forceps. Epidermis so prepared was minced with scissors and homogenized in 50 mM Tris, pH 7.6, with 6 M urea and 1 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was stirred at 4°C for 1 h and then centrifuged at 100,000 *g* to obtain a clear supernatant.

This extract which contained 300–350 mg protein was applied to a 2.5 × 35 cm column of DEAE-cellulose at a flow rate of 30 ml/h. The column was washed with extraction buffer and the initial 280-nm absorbing peak which contained 25–50 mg of protein was pooled. The partially purified filaggrin was adjusted to pH 8.3 and applied to a 1.1 × 5 cm column of QAE-Sephadex. The column was washed with starting buffer and then the filaggrin was eluted with starting buffer containing 50 mM NaCl. Approximately 3 mg of protein was recovered. This was concentrated to about 3 ml by pressure filtration against an Amicon PM-10 membrane and then prepared for SDS-PAGE by making the solution 0.0625 M in Tris, pH 6.8, 2% in SDS and 10% in glycerol, and heating at 100°C for 3 min. Samples were electrophoresed in a 3 mm-thick 10% polyacrylamide SDS gel. The strip of gel containing a polypeptide with the mobility of human filaggrin was excised, and the purified protein was electroeluted using the ISCO model 1750 sample concentrator and the instructions supplied by the manufacturer.

Rat filaggrin was purified as described by Harding and Scott [12] with a final purification by preparative SDS-PAGE. The protein was homogeneous by both 1- and 2-dimensional electrophoresis.

Preparation of Antibodies

A monoclonal antibody to human filaggrin was generated by standard techniques involving immunization of Balb/c mice, removal of the spleen, and recovery of antibody producing B-lymphocytes followed by fusion of these cells with NS-1 mouse myeloma cells to produce long-lived antibody producing hybrid lines. Briefly, mice were given 3 i.p. injections of 20–30 μg of filaggrin in Freund's incomplete adjuvant with 2 weeks between injections. One week after the third injection, antifilaggrin activity in the mouse sera was detected by an enzyme-linked immunosorbent assay (ELISA) technique. Mice were rested for 6 months and then given a fourth injection of filaggrin. After 2 additional weeks, mice were given 3 injections at 1-day intervals and sacrificed 1 day after the final injection. Fusions were carried out with 90 × 10^6 spleen cells and 15 × 10^6 NS-1 cells in a volume of 0.35 ml of 37% polyethylene glycol in DMEM for 3–5 min. Cells were plated in 96-well cloning racks containing 5000 mitomycin C-treated 3T3 cells per well. Wells were maintained for 2 weeks on DMEM (high glucose) containing 12% FCS supplemented with 10^{-4} M hypoxanthine, 4 × 10^{-7} M aminopterin, and 1.6 × 10^{-5} M thymidine (HAT). They were then weaned onto HAT containing no aminopterin (HT), and finally onto DMEM containing 12% FCS. At 75% of confluent density culture medium was withdrawn and tested for reactivity to human filaggrin. Those wells that contained an antibody were then cloned by limiting dilution. Two antibody-producing clones were derived and one of these (HF-1) was selected for study.

Antisera were raised in rabbits by injection of 1 mg of purified rat filaggrin in Freund's complete adjuvant. Booster injections were given at 2-week intervals. The antisera were purified with affinity columns of filaggrin coupled to CNBr-activated Sepharose 4B.

RESULTS

In a prior publication it was shown that an antibody raised to rat filaggrin reacted with extracts of cultured rat epidermal cells. Immunoblotting revealed several cross-reactive polypeptides and included filaggrin and polypeptides of higher and lower M_r [14]. In these experiments no reactive components that corresponded to profilaggrin were seen. Due to the well-documented susceptibility of profilaggrin to proteolysis it was decided to repeat these experiments using methods more suited to the retention and detection of this protein. Cultures were washed quickly with ice-cold PBS and then homogenized rapidly in 10 mM Tris, pH 8.0, containing 9.5 M urea, 2 mM PMSF, 25 mM dithiothreitol, and 2 mM EDTA. Samples were made 2% in SDS and heated at 100°C for 3 min.

These extracts were run in SDS gradient polyacrylamide gels as described earlier [12]. As can be seen in Fig 1*A*, a polypeptide is visible in cell extracts which corresponds to the profilaggrin band seen in extracts of newborn rat epidermis. The identity of this polypeptide was confirmed by immunoblotting as shown in Fig 1*B*. The antibody used was an affinity-purified antirat filaggrin. It can be seen that strong reactions occur not only with the profilaggrin of epidermis but also with a polypeptide of similar mobility present in cultured rat cell extracts. It should also be noted that several additional components in both epidermis and cultured cells react with the antibody. A number of components with M_r lower than filaggrin are seen in epidermis but not in cultured cells. Additionally, it can be seen that both profilaggrin and a band corresponding to filaggrin are visible in extracts of cultured keratinocytes at different lengths of time in culture. These studies do not permit us to distinguish be-

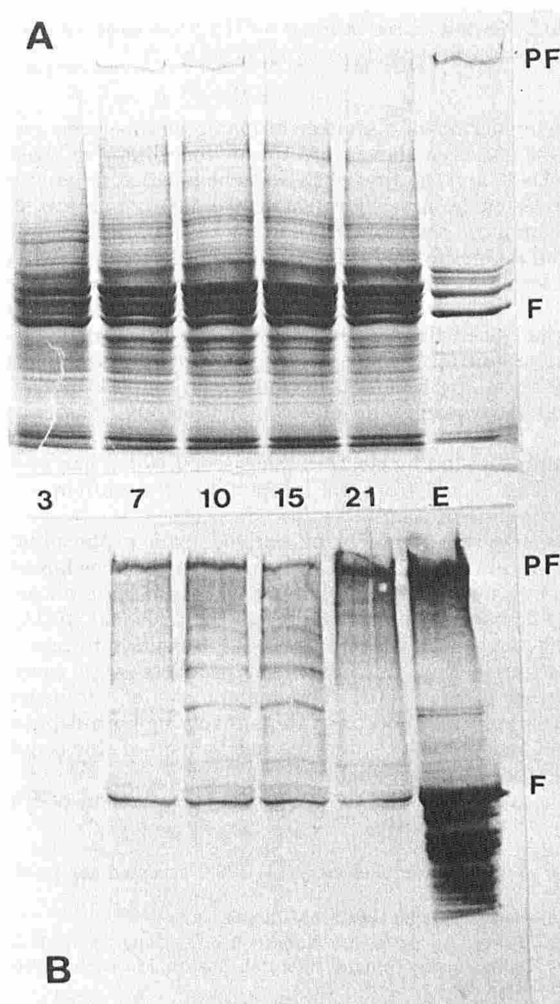

FIG 1. Separation and detection of rat filaggrin. SDS electrophoresis and immunoblotting. Protein extracts were made in 10 mM Tris HCl, pH 8.0, containing 9.5 M urea, 2 mM EDTA, 2 mM PMSF, and 25 mM dithiothreitol. (*E*) denotes extracts of newborn rat epidermis. Extracts of cultured keratinocytes are in tracks marked *3, 7, 10, 15,* and *21.* The numbers refer to the culture age (days) of the cells when extracted. *F* and *PF* mark the positions of filaggrin and profilaggrin. *A*, Separation of proteins on a 3–15% gradient SDS gel. *B*, Immunoreplica of (*A*) using the affinity-purified antibody to rat filaggrin.

tween true filaggrin and a phosphorylated profilaggrin fragment [6] of similar M_r, so we cannot be certain that processing has reached the filaggrin stage.

To further confirm the identity of profilaggrin, cell culture extracts were examined by 2-dimensional electrophoresis and compared to those of epidermis. In these experiments a cultured cell polypeptide with the same isoelectric point and M_r as epidermal profilaggrin was seen (Fig 2).

Human epidermal filaggrin with an M_r of 37,000 was purified to homogeneity (Fig 3). Fig 4 shows an SDS extract of human

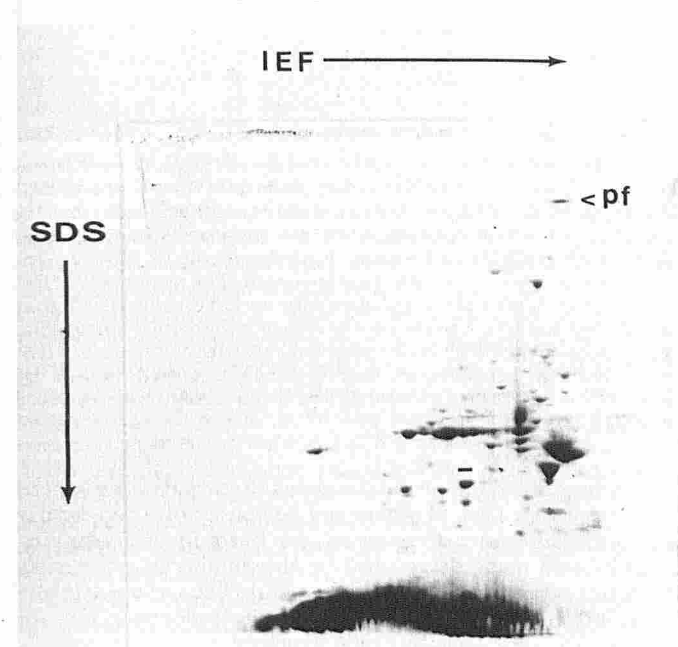

FIG 2. Two-dimensional electrophoresis of keratinocyte proteins. Confluent rat keratinocytes were extracted in 9.5 M urea and examined by the two-dimensional electrophoresis using non-equilibrium pH gradients in the first dimension and (3–15%) gradient SDS gels in the second dimension. *pf* indicates the spot which superimposes on profilaggrin of newborn rat epidermis.

FIG 3. Isolation of human filaggrin. SDS gel showing the original urea extract of human leg epidermis (*1*) and the final preparation of human filaggrin (2) isolated by ion exchange chromatography and preparative SDS electrophoresis.

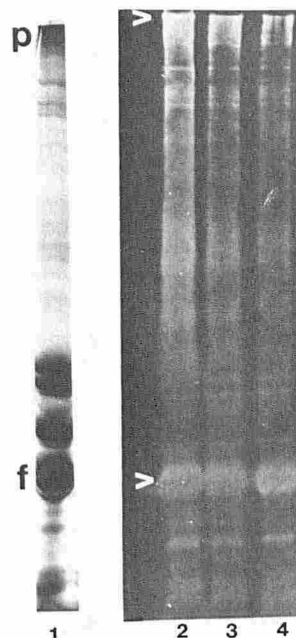

FIG 4. Reaction of HF-1 with human foreskin proteins. An SDS gel showing foreskin epidermal proteins extracted with 50 mM Tris pH 8.1, containing 2% SDS, 2 mM EDTA, 1 mM PMSF, and 10 mM dithiothreitol is seen in *1*. Immunoreplicas of (*1*) are seen in *2*, *3*, and *4*, with *3* and *4* containing one-half and one-fourth the protein of (*2*), respectively. *p* Indicates profilaggrin, *f* denotes filaggrin, and *arrowheads* denote these on the immunoreplicas.

foreskin epidermis immunoblotted with HF-1. The antibody reacts strongly with a band with the mobility of filaggrin. A number of other bands are also present and one of these has a $M_r > 300,000$. Profilaggrin would be expected to occur in this extract and electrophorese at this position. The reaction of HF-1 to isolated filaggrin can be seen in Fig 5.

HF-1 was also reacted with immunoblots of SDS extracts of cultured human keratinocytes. These extracts also contained a component with a $M_r > 300,000$ as well as a diffuse band of somewhat lower M_r (Fig 5). When cultured cells were extracted in 50 mM Tris, 2 mM EDTA, and 1 mM dithiothreitol and incubated at 37°C for 30 min prior to equilibration in SDS buffer, the immunoblots gave a different reaction pattern. The reaction of HF-1 to the high-M_r material was markedly decreased (Fig 5, track 6) and lower-M_r components became more visible. In a few experiments a very faint area of reactivity was produced at the filaggrin position (data not shown). It should be noted that the protein staining pattern of the incubated and unincubated sample were essentially identical, indicating that the amount of filaggrin-related proteins was very low. Only at the profilaggrin position could a slight loss of protein be observed. The same results were obtained with 2nd to 10th subcultured cells and in cells maintained in culture for 1–3 weeks.

HF-1 was absorbed with purified human filaggrin and then reacted with immunoblots of the extracts of leg and human foreskin epidermis and cultured human keratinocytes. No fluorescent bands were seen, while in control experiments using the unabsorbed antibody the fluorescent bands described above were observed.

HF-1 was reacted with human epidermis and found to stain the granular layer. Its localization within this layer was primarily in the granules themselves although some staining of the cytoplasm was also visible (Fig 6 *a,b*). No reactions were seen in the basal layers. In epidermis in which the granule-containing cells were decreased or absent such as in ichthyosis vulgaris or psoriasis, the reaction of HF-1 was spotty and corresponded to the few granular cells visible in hematoxylin

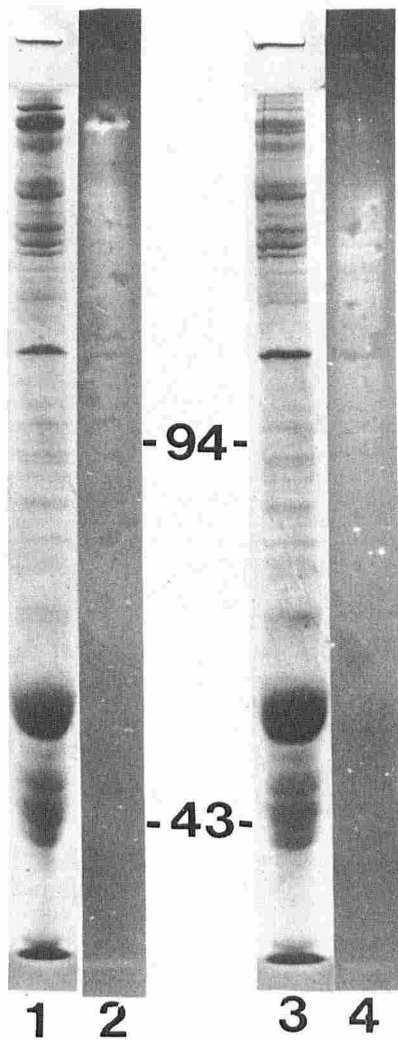

FIG 5. Reaction of HF-1 with proteins of cultured human keratinocytes. Confluent human keratinocytes were homogenized in 50 mM Tris pH 8.1 containing 2 mM EDTA. One aliquot was immediately made 2% in SDS and 1 mM in PMSF. This sample is shown in *1* and its immunoreplica is seen in *2*. A second aliquot was allowed to stand at 25°C before adding SDS and PMSF. This sample is shown in *3* and its immunoreplica in *4*.

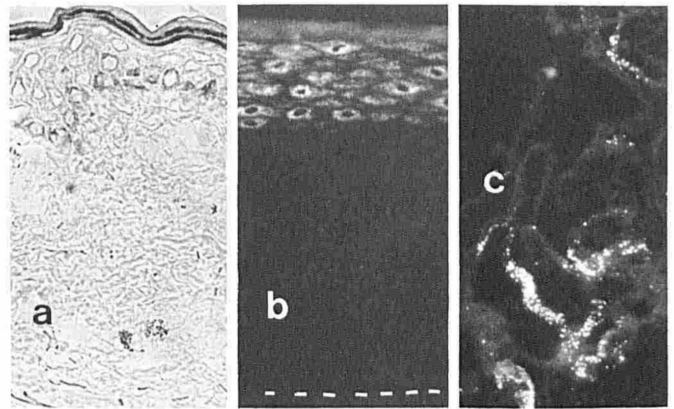

FIG 6. Reaction of HF-1 with human epidermis and cultured keratinocytes. *a*, Indirect immunoperoxidase reaction of normal epidermis with a 1:100 dilution of HF-1. After re-reactivity with an avidin-biotin horseradish peroxidase complex, color was developed by incubating with hydrogen peroxide and 3-amino-9-ethylcarbazole (× 313). *b*, Indirect immunofluorescence with thick epidermis. The section was first reacted with undiluted HF-1 and then with an FITC-conjugated goat antibody to mouse IgG. The *dashed line* indicates the dermal-epidermal junction (× 480). *c*, Indirect immunofluorescence with cultured keratinocytes. Confluent keratinocytes were detached as a single sheet by lifting with forceps. Sheets were rolled and frozen sections were reacted as in (*b*) (× 540).

and eosin-stained sections. When the antibody was reacted to cultured human keratinocytes its reaction was granular in appearance and seen in only some of the cells (Fig 6*c*). HF-1 did not react with newborn rat epidermis by indirect immunofluorescence. Moreover, the antirat filaggrin did not cross-react with human epidermis.

DISCUSSION

Although the functions of filaggrin have not been completely defined, the ability of these proteins to cause aggregation of keratin filaments in vitro suggests a similar activity in vivo. The continuous breakdown of filaggrins in stratum corneum suggests that while they may be necessary for the initial formation of keratin macrofibrils they are not required for their maintenance. The occurrence of filaggrin-related discrete proteins of varying M_r was recognized in the earliest studies of keratohyaline proteins [1] and suggested they underwent a rapid turnover. The rapid breakdown of the filaggrins has now been firmly established by pulse chase experiments utilizing [^{3}H]histidine [12,19].

The ability to be rapidly metabolized may be a consequence of the unique structure of the filaggrins. Recent data have led to the suggestion that filaggrins are composed of a repeating polypeptide unit, with the size of the filaggrin or filaggrin-related protein being determined by the number of repeating polypeptides that are linked together [20]. Thus, the multiple but distinct bands often seen by SDS-electrophoresis are in agreement with the repeating unit structure.

Not all the filaggrin-related proteins observed in epidermis were detectable in cultured cells using conditions that gave maximum growth of cells. This is true for both rat and human cells. In rat epidermis and cultured cells, profilaggrin and a series of related products of lower M_r were present, while cultured cells did not show polypeptides smaller than filaggrin as was seen in epidermis. On the other hand, human cultured cells showed mainly profilaggrin and high-M_r degradation products, a different pattern than observed with epidermis.

The decrease in observable cross-reactive bands in culture is most likely due to an actual absence of filaggrin metabolites. It is unlikely that some filaggrin-related proteins are present but not detectable by the antibodies. The proposed structure of the filaggrins is such that most if not all epitopes will be contained in a repeating polypeptide and this polypeptide will be present in filaggrin, profilaggrin, and all other related proteins. It would be expected that antibodies reacting with high-M_r filaggrins would also react with any smaller filaggrin fragments that contain the basic unit. Therefore, in the cultured keratinocytes of both rat and human the lower number of reacting bands indicates that fewer filaggrin-related fragments are present.

The decreased level of filaggrin or filaggrin-related proteins in culture could reflect decreased amounts of filaggrin production by the cells, decreases in the number of cells producing filaggrin, or increases in the rate of filaggrin breakdown by the cells. The incubation experiment in which human profilaggrin is allowed to be processed shows only breakdown products. This suggests that the bulk of cells present is capable of only limited processing of profilaggrin. Since no small products are formed, the experiment further suggests that the lack of filaggrin is not due to its formation and then rapid breakdown. A more likely possibility is that the processing of profilaggrin becomes more complete as the keratinocyte becomes more differentiated. Keratinocytes in culture which are less differentiated than those of epidermis may not have the ability to process profilaggrin

completely. This possibility is reasonable since keratinocytes in culture do not form a stratum corneum, the in vivo site of filaggrin breakdown. If the ability to process the filaggrins is a function of differentiation then those cells that become differentiated enough to produce filaggrin may become detached from the culture. It has been established that shedding of the most differentiated cells from the culture surface occurs in medium containing FCS [21]. The shedding is increased [22] when vitamin A is added to the medium and decreased [21] when vitamin A is removed from the medium. Since both the rat and human keratinocytes were cultured in medium containing undelipidized FCS, differentiating cells undoubtedly were shed from the culture. Had these cells remained adherent they might have acquired the ability to accumulate filaggrin.

In cultured rat keratinocytes, the processing of profilaggrin is more complete since a polypeptide of the approximate M_r of filaggrin is plainly visible. The lack of filaggrin breakdown products in culture is a major difference from the in vivo situation. The line of newborn rat cells have retained their capacity to differentiate and are capable of forming a stratum corneum-like structure when grown on a collagen matrix [14]. The cells differ from human keratinocytes in that they remain viable in culture for an extended period of time and require no feeder layers or exotic supplements for growth. Because rat and human cells behave so differently in culture it is not surprising that the rat cells should show a higher degree of profilaggrin processing. However, as in the human, not as many cross-reactive species are seen in culture as are visible in epidermis. This parallel to the human situation also may be explained by the shedding of cells before they are able to completely metabolize filaggrin so that the cells that remain adherent to the culture have not processed the profilaggrin to polypeptides smaller in M_r than filaggrin.

The results and observations presented in this paper suggest methods by which profilaggrin processing may be studied. The production of filaggrins may be monitored in well-described culture systems using the affinity-purified antibody to rat filaggrin or HF-1, the monoclonal antibody to human filaggrin. Culture conditions may be altered and the effects of these alterations on the program of profilaggrin synthesis and processing may be conveniently studied.

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