Identification of Two Intermediates during Processing of Profilaggrin to Filaggrin in Neonatal Mouse Epidermis

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ABSTRACT A major event in the keratinization of epidermis is the production of the histidine-rich protein filaggrin (26,000 mol wt) from its high molecular weight (>350,000) phosphorylated precursor (profilaggrin). We have identified two nonphosphorylated intermediates (60,000 and 90,000 mol wt) in NaSCN extracts of epidermis from C57/Bl6 mice by in vivo pulse-chase studies. Results of peptide mapping using a two-dimensional technique suggest that these intermediates consist of either two or three copies of filaggrin domains. Each of the intermediates has been purified. The ratios of amino acids in the purified components are unusual and essentially identical. The data are discussed in terms of a precursor containing tandem repeats of similar domains. In vivo pulse-chase experiments demonstrate that the processing of the high molecular weight phosphorylated precursor involves dephosphorylation and proteolytic steps through three-domain and two-domain intermediates to filaggrin. These processing steps appear to occur as the cell goes through the transition cell stage to form a cornified cell.

Mammalian epidermis is a stratified tissue with dividing basal cells overlaid by a series of differentiating cells proceeding from spinous cells to granular cells, and culminating in the dead cells of the stratum corneum. A histidine-rich protein called filaggrin has been isolated from stratum corneum (1, 2). It appears to function as a matrix protein between the keratin filaments within the fully differentiated cells of the stratum corneum (3). Antibodies to filaggrin localize in the stratum corneum and in the keratohyaline granules of the less differentiated granular cells (4). Ultrastructural analyses show [^{3}H]histidine preferentially incorporated into the granular layer where keratohyaline granules are being produced (5). Pulse-chase experiments with [^{3}H]histidine show label in a high molecular weight precursor (>350,000) which is chased into filaggrin (6–9). The high molecular weight precursor is highly phosphorylated, while filaggrin is not (10). Electron-probe microanalysis has demonstrated high levels of phosphate in keratohyaline granules, while the stratum corneum has virtually none (11). These data are interpreted to suggest filaggrin is made as a highly phosphorylated precursor, stored in the keratohyaline granules, and then dephosphorylated and proteolytically processed to form the matrix protein, filaggrin, during cornification of the cells. The large phosphorylated precursor is called profilaggrin as suggested by Harding and Scott (12).

Both the synthesis and processing of this large molecular weight phosphorylated precursor are complex multistep processes that are probably tightly controlled by the cell. This paper addresses only the processing events that yield filaggrin. We have isolated two intermediates that appear to contain two and three copies of a filaggrin domain, and which we call 2DI and 3DI, for two-domain intermediate and three-domain intermediate.

MATERIALS AND METHODS

Extraction of Tissue: We obtained epidermis from newborn C57/B16 mouse skin (0–2 d) by heating the skin for 3 min at 50°C in PBS containing 10 mM EDTA and then chilling it in ice-cold EDTA-PBS and peeling the epidermis from the dermis. Typically 30–35 mg of tissue was obtained from each animal. The skins were homogenized in a tissue homogenizer in ice-cold 1.0 M NaSCN, 50 mM HEPES, 10 mM EDTA, pH 6.8, containing 0.3 mM orthophenanthroline, 20 μg/ml phenylmethylsulfonyl fluoride, and 0.1% isopropanol. Filaggrin and its precursors were precipitated from the supernatants by dilution into 10 vol of ice-cold water and centrifugation (0°C) at 12,000 *g*. Alternatively, tissue was extracted in 8 M urea, 10 mM EDTA, 50 mM Tris, pH 8, containing the same concentration of inhibitors. In either case, homogenates were centrifuged at 27,000 *g* for 30 min at 0°C. Supernatants were either kept on ice for use on the same day or frozen at −20°C for later use.

SDS PAGE and Western Blotting: Samples were dissolved and analyzed on SDS PAGE by the method of Laemmli (13) except that gradient gels from 4–16% acrylamide were used. Inclusion of 0.15 M NaSCN did not interfere with the electrophoresis. The upper running buffer contained 1 mM Na thioglycolate to scavenge free radicals and oxidants in the gel (14). Acid

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urea gels (pH 2.3) were run as in Brewer and Ashworth (15), except that running buffer contained 2 mM cysteine as a savenger. Gels were stained with Coomassie Brilliant Blue in 10% acetic acid, 50% methanol and destained in the same buffer. Low molecular weight standards (Pharmacia Inc., Piscataway, NJ) were supplemented with 200,000-mol-wt myosin (16) and 335,000-mol-wt thyroglobulin (17).

Antigenic proteins were analyzed by Western blotting onto nitrocellulose followed by reaction with the peroxidase/antiperoxidase procedure (18). Excess binding sites were blocked with 3% BSA, and then blots were incubated successively with rabbit antibody to rat filaggrin, goat anti-rabbit IgG, and a rabbit antiperoxidase-peroxidase complex. Peroxidase was visualized with 3,3'diaminobenzamidine and H_2O_2.

In Vivo Radiolabeling: Histidine-rich proteins were labeled in vivo with 15 μl of a solution containing either 15 μCi of [^{3}H]histidine (ICN Corp., Irvine, CA) or 150 μCi of [^{32}P]phosphate (New England Nuclear, Boston, MA) injected subcutaneously into the upper back of newborn or 2-d-old mice. Animals were returned to their mothers. The histidine label was chased at 2 h by injecting 15 μl of 1 mM histidine near the site of the first injection. At various times animals were killed by cervical dislocation, and the skins were removed and extracted with NaSCN as described above. Three litters were used for each experiment and two to three animals were taken for each time point.

10 μl of supernatant from each time point was added to 90 μl of Laemmli's sample buffer, boiled 3 min, and analyzed by gradient SDS PAGE. In the phosphate labeling experiments, autoradiography was done with dried gels and XOMAT film (Kodak, Rochester, NY) at room temperature. In the histidine labeling experiments, stained gels were treated with ENHANCE (New England Nuclear) for fluorography using XOMAT film at −70°C. Western blots of labeled proteins were sprayed with Enhance for fluorography.

Peptide Mapping: To compare proteins in extracts, we developed a method of peptide mapping by a two-dimensional modification of the technique of Cleveland et al. (19). A similar method has been used by Bordier and Crettol-Jarvinen (20). In this procedure, after a preliminary SDS PAGE separation, proteins in an entire lane were exposed to proteases during electrophoresis at right angles in a second SDS PAGE. First, the proteins (precipitated by dilution in water) were resolved on a 4–16% gradient in SDS PAGE as described above. The gel was stained for 10 min in 1% Coomassie Blue in water and destained in water just until lanes could be clearly seen (~20 min). The whole lanes were cut out and placed in Laemmli's sample buffer containing 15% glycerol instead of the usual 5%, for 15–20 min. Each strip was then placed horizontally in a slot above a 1-cm stacking gel on top of a second SDS PAGE gel (5–20% gradient). As noted by Tÿssen and Kurstak (21), variations in length of the stacking gel caused changes in the degree of proteolysis. Insertion of the strips was facilitated when the first gel was 1.2-mm thick and the second gel was 1.5-mm thick. A small amount of the sample buffer (containing 15% glycerol) was added to assure a level surface for the subsequent addition of 200 μl of a protease solution on top of the gel strip. *N*-tosyl-L-phenylalanine chloromethyl ketone–treated chymotrypsin, generously provided by Dr. K. Titani (University of Washington), was dissolved in 5% glycerol, 50 mM Tris (pH 6.8), 0.01% bromphenol blue just before use. The most reproducible results were obtained when electrophoresis was not stopped at the stacking interface. The initial current was 50 mA in a gel 13-cm wide. When the bromphenol blue had passed through the sample strip, the current was adjusted to 35 mA.

Purification of Filaggrin, Two- and Three-Domain Intermediates, (2DI and 3DI),[1] *and Profilaggrin:* Proteins in NaSCN extracts from 10 skins were precipitated by adding 10 vol of distilled water and chilling in an ice bath for 5 min before centrifugation (12,000 *g* for 30 min). The pellet was extracted with 30 ml of 10% formic acid at room temperature; acid-soluble proteins were lyophilized, taken up in 10 ml of 6 M urea, 10 mM EDTA, 50 mM Tris, pH 8, and applied to DEAE-sepharose as in Dale (1). The high molecular weight precursor bound to the column and was eluted later with 250 mM NaCl in the equilibration buffer. Filaggrin, 2DI, and 3DI did not bind to the column. This unadsorbed mixture of proteins was desalted into 10% formic acid on a column of Bio-Gel P2, lyophilized, dissolved in 0.3 ml of 10% formic acid (adjusted to pH 2 with NaOH), and separated from insoluble material by centrifugation. The supernatant was applied to a column of Bio-Gel P200, 200–400 mesh (0.7 × 91 cm) equilibrated in 10% formic acid, pH 2. The protein was eluted at room temperature at 1.2 ml/h. An aliquot of each fraction was lyophilized, taken up in sample buffer, and analyzed by SDS PAGE in order to locate the proteins of interest.

Amino Acid Analysis: Samples of 3DI, 2DI, and filaggrin for amino acid analysis were first dialyzed against 1% formic acid at 3°C for 8 h using Spectropore tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) washed with hot EDTA/$NaHCO_3$. Profilaggrin was desalted into 10% formic acid on Bio-Gel P2. Aliquots were lyophilized and hydrolyzed in 6 N HCl at 106°C for 22 h before analysis on a Dionex D-500 amino acid analyzer (Dionex Corp., Sunnyvale, CA). No corrections were made for destruction of serine. Low levels of amino acids (<0.2 nmol) were detected in blanks and were subtracted from the analyses. Analyses were repeated at two different concentrations and normalized via alanine.

[1] *Abbreviations used in this paper:* 2DI and 3DI, two- and three-domain intermediates, respectively.

RESULTS

Extraction of Filaggrin-like Proteins

Urea extracts of epidermis show a number of proteins that interacted with antibody to filaggrin and several that did not (Fig. 1, lanes *1* and *3*). Since keratins contaminate this urea extract, an extraction method with NaSCN was developed which left the keratins in the tissue (Fig. 1, lanes *2* and *4*). This had the additional advantage that filaggrin-related proteins are selectively precipitated by dilution with 10 vol of water. To check that all of the proteins of interest were extracted by this new method, we performed Western blots with antibody to filaggrin. Although the antibody used has some reactivity with nonfilaggrin proteins in the extract (e.g., the prominent 200,000-mol-wt band in Figs. 1 and 4), the blotting experiments clearly show the same cross-reactive proteins were extracted in NaSCN as in the traditional 8 M urea buffer. Preimmune serum also reacted with the 200,000-mol-wt protein, which was shown to be unrelated to filaggrin by peptide mapping.

The relative selectivity of the NaSCN extraction procedure was confirmed by composition studies (Table I). The precipitate has very nearly the same amino acid composition as the unusual one that characterizes pure filaggrin (2). For example, there is a high content of arginine, histidine, and serine, but none of the cystine or methionine characteristic of keratins.

Time Course-labeling Studies

Using [^{3}H]histidine in a pulse-chase, in vivo labeling experiment, we examined the order of appearance of these proteins in newborn mice. The results of a 44-h time course are shown in the fluorograph in Fig. 2 and by densitometric analysis in

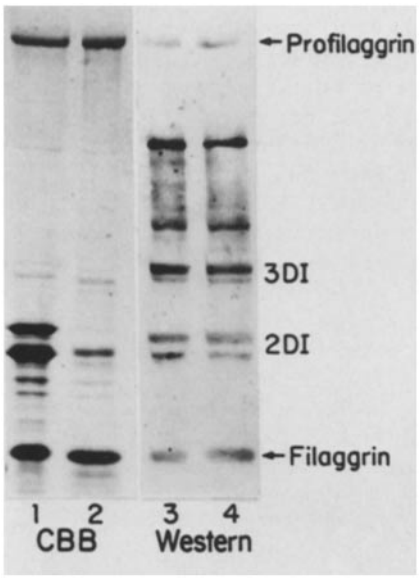

FIGURE 1 SDS PAGE of extracts of newborn mouse epidermis. Lanes *1* and *3* are urea extracts; lanes *2* and *4* are NaSCN extracts. Lanes *1* and *2* are stained with Coomassie Brilliant Bue (*CBB*); lanes *3* and *4* are Western blots using antibody to rat filaggrin.

TABLE I

Mole Percent of Amino Acid Residues in Acid Hydrolyzates of Filaggrin-related Proteins

	Precipitate*	Profilaggrin	3DI	2DI	Filaggrin	Filaggrin‡
Asx	5.4	6.3	4.4	5.3	6.0	5.2
Thr	1.8	1.3	Trace	Trace	Trace	0.4
Ser	15.3	15.6	18.3	19.2	19.6	20.4
Glx	19.1	21.2	22.8	22.1	21.9	20.6
Pro	3.25	2.8	6.7	3.4	3.1	3.1
Gly	15.1	17.8	19.5	17.7	17.7	17.0
Ala	8.8	7.8	6.1	8.6	9.1	8.6
Val	3.4	3.0	2.0	2.5	2.5	3.0
Met	0.1	0.4	—§	—	—	0.1
Ile	0.54	1.8	—	—	—	0.2
Leu	1.55	2.4	—	—	—	0.7
Tyr	1.74	0.2	—	—	—	0.6
Phe	1.02	0.9	—	—	—	0.6
His	7.4	8.9	8.4	8.5	8.9	8.4
Lys	1.3	1.1	—	—	—	0.1
Arg	11.1	9.2	12.0	12.7	11.4	11.5

* Material that precipitates from the 1 M NaSCN extract upon 10-fold dilution in water.
‡ Steinert et al., reference 2.
§ None detected (<0.2).

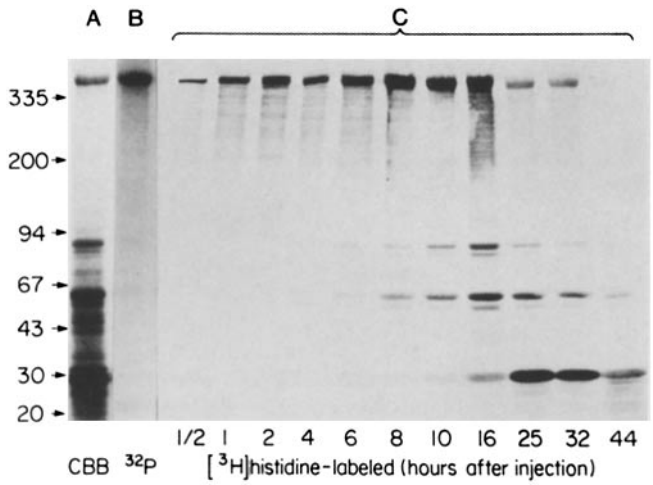

FIGURE 2 SDS PAGE of epidermal NaSCN extracts of newborn mouse skin from in vivo pulse-chase experiments. (*A*) Proteins stained with Coomassie Brilliant Blue (*CBB*). (*B*) ^{32}P-labeled proteins, precipitated from a 16-h NaSCN extract by 10-fold dilution. 19 other timed samples (not shown) gave virtually identical results. (*C*) time course of [^{3}H]histidine-labeled proteins. Time from injection of label is indicated under each lane. Longer exposure showed no label in intermediates until 4 h. Values at left equal molecular weight × 10^{-3}.

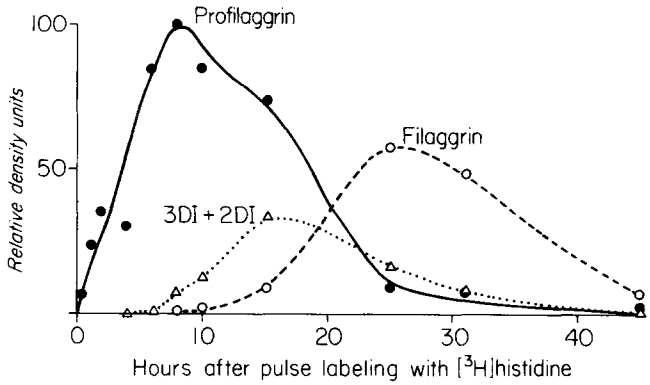

FIGURE 3 Densitometric data derived from the in vivo [^{3}H]histidine pulse-chase experiment in Fig. 2*C*. 100 density units correspond to 3,100 cpm.

Fig. 3. Label was first seen in a high molecular weight precursor (>350,000). By 4 h, label was detected in two pairs of bands at approximately 90,000 (hereafter referred to as 3DI) and 60,000 mol wt (2DI). The differences between the major and minor bands of each pair is not clear. Because of the similar kinetics of appearance and disappearance of label in all four bands, we grouped them together in the densitometric analysis of Fig. 3. After 8–10 h the label was first evident in filaggrin. The timing of the first detection of label was similar in three experiments. Newborn animals showed virtually complete disappearance of label from the high molecular weight precursor by 35 h, whereas 2-d-old animals required ~50 h. These data are consistent with a precursor-intermediate-product relationship from the >350,000-mol-wt precursor (profilaggrin) through intermediates (3DI and 2DI) to the mature product, filaggrin.

Most of the incorporated radioactivity is found in filaggrin-related proteins. The efficiency of uptake of the label was relatively uniform from animal to animal. In one experiment using three litters (27 animals), total incorporation was 1.05 × 10^6 cpm/min per animal (SD = 0.18). After 80 h, fewer total counts were recovered, presumably owing to sloughing of dead cells. After acid hydrolysis of samples at 1 and 24 h, all label was found in histidine. After 45 h, label began to appear in trichloroacetic acid–soluble material which elutes with urocanic acid on thin-layer chromatography (J. Peschon and K. Resing, unpublished data). Of the incorporated counts, 70% (0.73 × 10^6 cpm per animal) was extracted into 1 M Na thiocyanate, 85% (0.62 × 10^6 cpm per animal) of that in 1-h extracts precipitated upon dilution (Table II). Gel slicing experiments showed at least 90% of the label in the precipitate was in profilaggrin.

Since the precursor (>350,000) is known to be phosphorylated (8, 10), similar pulse-chase experiment was done with [^{32}P]phosphate to see if any of the intermediates were also phosphorylated. In 20 samples collected over a 2-d period, only the high molecular weight precursor was labeled. Fig. 2,

lane *B*, illustrates one such analysis. No phosphate was detected in the 2DI, 3DI, or filaggrin bands.

Isolation and Characterization of 3DI, 2DI, and Filaggrin

The major filaggrin-related proteins were precipitated by 10-fold dilution of the thiocyanate extract in ice-cold water. At this stage of purification, they appeared to co-precipitate with a contaminant, possibly chromatin, because the purified proteins are soluble in dilute thiocyanate. The precipitate was dissolved in 6 M urea, 50 mM Tris, pH 8, and applied to DEAE-Sepharose.

Fig. 4 shows that filaggrin and the intermediate forms, 2DI and 3DI, did not bind to DEAE-Sepharose at pH 8. These unbound proteins could be partly purified on Bio-Gel P200 in 10% formic acid (Fig. 5). A 25-h time point was subjected to gel filtration on P300. 0.33×10^6 cpm per animal was recovered in filaggrin, 0.10×10^6 cpm per animal in 2DI and 3DI, and 0.03×10^6 cpm per animal in profilaggrin (Table II). These data suggest that 20–25% of the label has disappeared during processing, which is in agreement with the densitometric analysis of fluorographs in Fig. 3.

TABLE II
Distribution of [^{3}H]Histidine Label in Extracts of Epidermis

Step	cpm per animal	Recovery
	$\times 10^6$	%
Labeled ≤2 h		
Incorporated in homogenate*	1.05 (0.18)	
1 M NaSCN extract (<2 h)*‡	0.73 (0.12)	100
Found in precipitates§	0.62 (0.09)	85
Labeled 25 h		
1 M NaSCN extract	0.80	100
Precipitate	0.53	66
Found in profilaggrin‖	0.03	4
Found in 3DI and 2DI‖	0.10	12
Found in filaggrin‖	0.33	41

Values in parentheses represent standard deviation.
* Estimated by counting aliquots of six homogenates.
‡ Soluble material in 1 M NaSCN.
§ Precipitated by 10-fold dilution in water. Gel slicing experiments showed that at least 90% was profilaggrin.
‖ Cpm recovered after 1 M NaSCN extract was analyzed by gel filtration. 18% of counts ran in the very small size range (<8,000 mol wt).

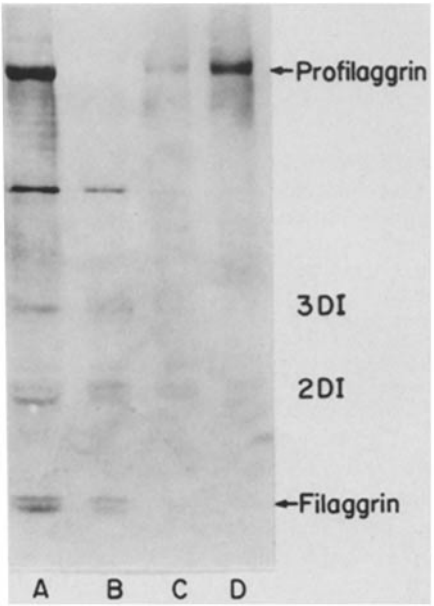

FIGURE 4 Western blot after SDS PAGE of fractions from DEAE-Sepharose (pH 8) chromatography (see Materials and Methods) using rabbit anti-rat filaggrin. (*A*) Sample as applied to column; (*B*) flow-through fraction containing 3DI, 2DI, and filaggrin; (*C*) fraction eluted by 100 mM NaCl; (*D*) fraction eluted by 250 mM NaCl (and used for amino acid analysis in Table I).

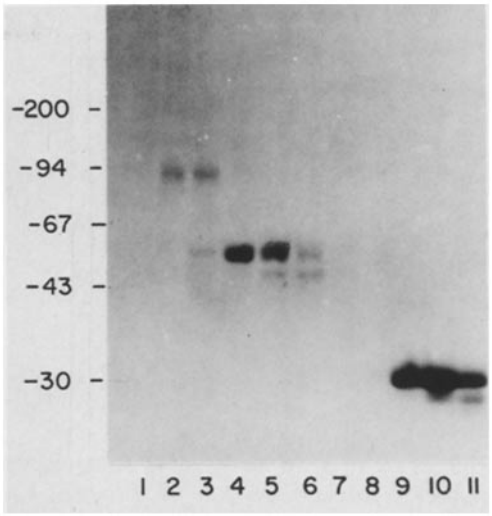

FIGURE 5 Filaggrin-related proteins separated according to their size. The mixture of proteins in the flow-through fraction of the DEAE-Sepharose column (Fig. 4, lane *B*) was separated on a Bio-Gel P200 column (see Materials and Methods) at 1.2 ml/h. Fractions collected hourly were examined by SDS PAGE and Coomassie Brilliant Blue staining. Lanes *1–11* correspond to fractions collected at 11 h through 21 h. Fractions sampled for amino acid analysis (Table I) correspond to lanes *2* (3DI), *4* (2DI), and *9* (filaggrin). Values at left are molecular weight $\times 10^{-3}$.

Amino acid analysis of the purest fractions (judged by SDS PAGE) showed that 3DI, 2DI, and filaggrin had nearly identical compositions (Table I). The slightly low serine content for profilaggrin is probably due to destruction of phosphoserine. No correction was made because control peptides showed various degrees of destruction of phosphoserine (from 10 to 40%). Chromatography on carboxymethyl-cellulose at pH 5 showed little resolution of the three components. Phosphate analysis by the method of Itaya and Ui (22) showed <0.5 phosphate/mol. This confirms the absence of phosphate in the intermediates as shown by in vivo [^{32}P]phosphate labeling experiments in Figure 2*B*. The elution behavior on DEAE- and carboxymethyl-cellulose suggest that the larger intermediates, 3DI and 2DI, are similar in net charge to filaggrin, a conclusion consistent with the absence of phosphate in all three proteins.

Two-dimensional Peptide Maps of Filaggrin-related Proteins

To determine the structural relationships among profilaggrin, filaggrin, and the intermediates, we performed peptide mapping using limited proteolysis during SDS PAGE by a modification of a method first described by Cleveland et al. (19). In this procedure, the proteins are first resolved by size on SDS PAGE in one dimension, then digested with a protease as they enter a second dimension of SDS PAGE. Undi-

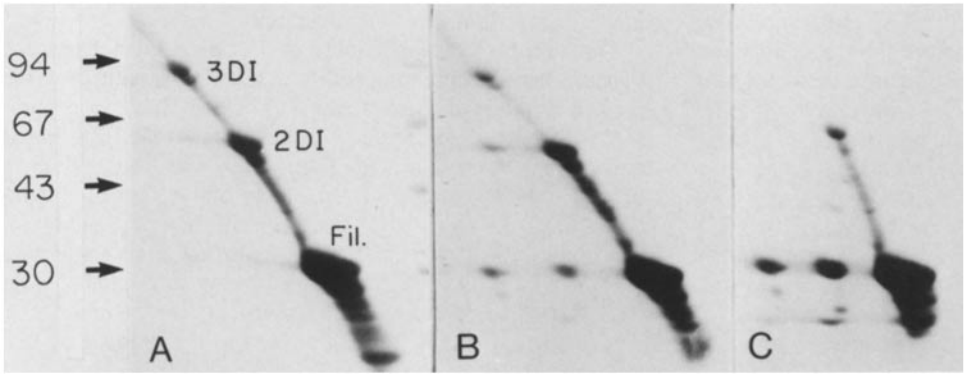

FIGURE 6 Two-dimensional limited chymotryptic peptide maps of 2DI and 3DI, suggesting domain substructure. In the first (horizontal) dimension, proteins precipitated by dilution of a NaSCN extract were first separated on SDS PAGE. In the subsequent second (vertical) dimension limited proteolysis by thermolysin in the stacking gel preceded separation (see Materials and Methods). The gel was stained with Coomassie Brilliant Blue. Chymotrypsin used was (*A*) 0.1 μg; (*B*) 1 μg; and (*C*) 10 μg. Values at left are molecular weight × 10^{-3}.

gested proteins are found on a diagonal, while peptides generated from each species are separated vertically. Using this technique, Lonsdale-Eccles et al. (8) showed that profilaggrin appeared to contain multiple copies of a phosphorylated form of filaggrin called φ, but the presence of keratins obscured analysis of the regions where 3DI and 2DI are now located. Fig. 6 illustrates that chymotrypsin cleaved 3DI to fragments co-migrating with 2DI or filaggrin, and cleaved 2DI to a peptide similar in size to filaggrin. In each case, the amount of mass, judged by densitometry of the Coomassie Blue staining of the original 3DI or 2DI regions, appeared similar to the mass of the proteolytically generated monomer. Taken together with the molecular weights, these data suggest that there are three filaggrin-like domains in each 3DI and two such domains in each 2DI. The domains appear to be bound together covalently since they do not dissociate in SDS PAGE in 6 M urea at pH 7.8 or 2.5 (data not shown). Limited proteolysis with chymotrypsin appeared to separate the domains, presumably in interdomain regions.

Upon more extensive digestion, profilaggrin, 2DI, and 3DI showed other differences (Fig. 7). Initially, each of the larger proteins was broken down to a filaggrin-sized peptide, from which peptides in the 20,000–24,000-mol-wt range were produced. Peptide A is similar in filaggrin, profilaggrin, and the intermediates. An offset spot just above A of 2DI is from the minor 60,000-mol-wt kd component. Peptide B is unique to profilaggrin, and peptide C appears to be unique to 2DI. These differences probably reflect prior in vivo proteolytic processing in interdomain linker regions. The possibility that linker peptides are removed during processing is supported by the observation that ~20% of the [^{3}H]histidine incorporated into profilaggrin was not recovered in 3DI, 2DI, or filaggrin.

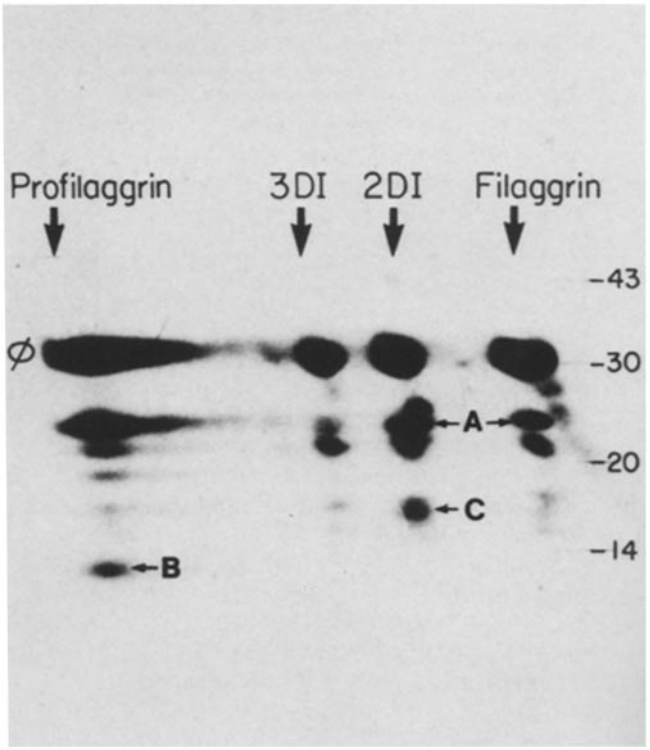

FIGURE 7 Fluorograph of a two-dimensional chymotryptic peptide map of proteins precipitated from a 1 M NaSCN extract of 12.5 mg of mouse epidermis labeled for 18 h with [^{3}H]histidine (65,000 cpm per gel). After the first separation (horizontal), a large amount of chymotrypsin (100 μg) was added to promote extensive proteolytic digestion and the products displayed in the vertical dimension. Similar peptides are seen in profilaggrin, 3DI, 2DI, and filaggrin.

DISCUSSION

In the work presented here, we have shown pulse-chase evidence that filaggrin is produced in vivo from a high molecular weight phosphorylated precursor, profilaggrin (>350,000), via 90,000- and 60,000-mol-wt protein intermediates. These intermediates have several properties in common with filaggrin. Most important, they have nearly identical, and quite unusual, amino acid compositions. In addition, they are all cationic, contain no phosphate, show limited size heterogeneity, and have similar peptide maps after limited proteolysis. They differ from filaggrin in their sizes, which are approximately twice or three times the molecular weight of filaggrin. These molecular weights do not change in 10% formic acid, in acid-urea-PAGE, in SDS PAGE, and in the presence of mercaptoethanol. Limited proteolysis suggests that the 90,000- and 60,000-mol-wt proteins consist of three and two repeats of a filaggrin domain, which provides additional support for the hypothesis that the largest precursor, profilaggrin (>350,000 mol wt), consists of many tandem repeats of a filaggrin unit (8, 24). The proposed polymeric structure for profilaggrin can be compared with examples of repeated copies of peptide hormones or to certain polyproteins, as found in viruses (cf. reviews in references 8 and 25). The polymeric profilaggrin is exceptional in the suggested length of the repeat size and in the larger number of copies of the repeat (at least 10).

Intermediates in the processing of filaggrin precursor have been observed before but the kinetics of labeling have not

been reported. Profilaggrin was also seen by Ramsden et al. (9), who labeled filaggrin ("band 2 protein") in vivo with a pulse of [^{3}H]histidine and observed label in a high molecular weight precursor ("350 K HRP") before that label appeared in filaggrin. A similar series of processing intermediates have also been observed in guinea pigs (26) and may be implied from work on rabbits (27) and humans (12). The unusual processing via intermediates such as seen in Fig. 2 has structural implications that must be accommodated in any polymeric model for profilaggrin. It is clear in Fig. 2*C* that 2DI and 3DI can be generated from the polymeric profilaggrin without generating much monomer (particularly in the 4–10-h range). Possibly the filaggrin domains are joined by two different types of bonds. If one of these sites is processed early (at 4–8 h) to produce 2DI and 3DI, and another later (10–20 h) to produce filaggrin, the pattern in Fig. 2*C* could be explained. Processing of the two types of sites could be controlled by timed release of proteases or by restricted access in the packed granule.

Profilaggrin is not only the species with the highest molecular weight, but also the *only* species that is phosphorylated and anionic. Therefore, dephosphorylation must be an early processing step. Because no phosphorylated intermediate was detected in the ^{32}P time course, dephosphorylation appears to precede proteolysis. Alternatively, the two events occur concomitantly, or certain aspects of processing may occur so rapidly that intermediates are not observed. It will be shown separately (Resing, K., B. Dale, and K. Walsh, manuscript in preparation) that some but not all of the phosphate may be removed as phosphopeptides during processing of profilaggrin to 3DI and 2DI. One can speculate that the removal of phosphate (or phosphopeptides) unmasks protease sites or that the presence of phosphate prevents premature interaction of cationic filaggrin with anionic keratin filaments, but such ideas have not yet been tested.

One can attempt to correlate the time course of the existence of the intermediates with the cellular events associated with this highly differentiated tissue. The relevant events occur between the granular cell stage and the fully differentiated stratum corneum cell, since profilaggrin is synthesized in the granular layer but only filaggrin is found in the stratum corneum (6, 7, 9, 10, 28). To correlate the cellular differentiation with the processing of profilaggrin (Fig. 3), estimates of the rate of upward movement of the cells is required. Since the granular layer is three to four cells thick, cells at various depths will undergo the transition at different times. Thus, profilaggrin made in the uppermost granular cell layer will reach the stratum corneum more quickly than that made in the lowermost layer. Fukuyama and Epstein (29) reported that granular cells labeled with [^{3}H]histidine begin to migrate into the lowermost layer of the stratum corneum by 6 h and that the migration is essentially complete in 24 h. The time scale may be compared with that of our [^{3}H]histidine-labeled cells, in which processing to 3DI and 2DI was first detected 3–4 h after the histidine pulse (Figs. 2 and 3) and was complete some 25–40 h later.

The experiments of Fukuyama and Epstein (29) also shed light on the physiological meaning of the 5–6-h lag time between the appearance of the intermediates and the appearance of filaggrin. The initial transport of label in their studies occurred into a transition cell, which we might now propose as the site of the enzymatic processing. 2DI and 3DI may appear early in such a transition, and the initial interaction with the keratins may occur at this stage. Subsequent production of filaggrin might then signal a condensation of the filaggrin-keratin macrofibril structures.

The eventual disappearance of histidine label from the filaggrin band occurs long before the label is sloughed off the surface of the skin. Scott et al. (30) believe filaggrin is completely degraded to free amino acids and that histidine is converted to urocanic acid. Filaggrin would thus appear to function as a "scaffolding" protein. In our system the breakdown to amino acids (and urocanic acid) begins at ~36 h (J. Peschon and K. Resing, unpublished observation), suggesting that the scaffolding is in place for ~30 h or during the passage through four to five cell layers. The lower layers of the stratum corneum show a progressive decrease in the amount of free sulfhydryl groups (31). Possibly the filaggrin scaffold is no longer required after the keratins have formed cystine disulfides.

The synthesis of a polymeric profilaggrin may be a common feature in mammalian epidermis. Studies of processing in this system have been complicated by the proteolytic lability of the profilaggrin and by the presence of massive amounts of keratins. The present use of high salt concentrations during extraction, and of the specific histidine label, together provide a clearer picture of events that occur in profilaggrin processing. These events constitute important cellular processes in epidermal differentiation. The relative ease with which processing can now be followed make it ideal for closer study of the cellular mechanisms of terminal differentiation. It remains to be seen whether modulation of these events from tissue to tissue, during disease states and during fetal development, may correlate with the type and extent of epithelial differentiation.

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