Cho, A. K., & Miwa, G. T. (1974) *Drug Metab. Dispos. 2*, 477.
Coombes, R. G. (1979) *Compr. Org. Chem. 2*, 305.
Denisov, F. T. (1974) *Liquid-Phase Reaction Rate Constants*, IFI Plenum Press, New York.
Duncan, J. D., & Cho, A. K. (1982) *Mol. Pharmacol. 22*, 235.
Duncan, J. D., Hallström, G., Paulson-Sörman, U., Lindeke, B., & Cho, A. K. (1983) *Drug Metab. Dispos. 11*, 15.
Duncan, J. D., Di Stefano, E. W., Miwa, G. T., & Cho, A. K. (1985) *Biochemistry* (preceding paper in this issue).
Elstner, E. F., & Heupel, A. (1976) *Anal. Biochem. 70*, 616.
Fee, J. A., & Valentine, J. S. (1977) in *Superoxide and Superoxide Dismutase* (Michelson, A. M., McCord, J. M., & Fridovich, J., Eds.), p 19, Academic Press, New York, NY.
Fischer, F., & Pfleiderer, G. (1922) *Z. Anorg. Allg. Chem. 124*, 61.
Jonsson, U., Lundkvist, G., Eriksson, S. O., & Lindeke, B. (1977) *J. Pharm. Pharmacol. 29*, 358.
Kono, Y. (1978) *Arch. Biochem. Biophys. 186*, 189.
Kono, Y., & Fridovich, I. (1983) *J. Biol. Chem. 258*, 13646.
Liebman, J. F., & Valentine, J. S. (1983) *Isr. J. Chem. 23*, 439.
Lindeke, B., Anderson, E., Lundkvist, G., Jonsson, U., & Ericksson, S. (1975) *Acta Pharm. Suec. 12*, 183.
Maynard, M. S., & Cho, A. K. (1979) *Proc. West. Pharmacol. Soc. 22*, 105.
Maynard, M. S., & Cho, A. K. (1981) *Biochem. Pharmacol. 30*, 1115.
Nanni, E. J., & Sawyer, D. T. (1980) *J. Am. Chem. Soc. 102*, 7591.
Nanni, E. J., Jr., Stallings, M. D., & Sawyer, D. T. (1980) *J. Am. Chem. Soc. 102*, 4481.
Rauckman, F. J., Rosen, G. M., & Kitchell, B. B. (1979) *Mol. Pharmacol. 15*, 131.
Rosen, G. M., & Rauckman, F. J. (1977) *Biochem. Pharmacol. 26*, 675.
Rosen, G. M., Finkelstein, E., & Rauckman, F. J. (1982) *Arch. Biochem. Biophys. 215*, 367.
Sawyer, D. T., & Gibian, M. J. (1979) *Tetrahedron 35*, 1471.
Sawyer, D. T., & Valentine, J. S. (1981) *Acc. Chem. Res. 14*, 393.
Sawyer, D. T., Calderwood, T. S., Yamaguchi, K., & Angels, C. T. (1983) *Inorg. Chem. 22*, 2577.
Sum, C. Y., & Cho, A. K. (1977) *Drug Metab. Dispos. 5*, 464.
Valentine, J. S., Miksztal, A. R., & Sawyer, D. T. (1984) *Methods Enzymol. 105*, 71.
Wajer, Th. A. J. W., Mackor, A., & DeBoer, Th. J. (1969) *Tetrahedron 25*, 175.

Multiple Copies of Phosphorylated Filaggrin in Epidermal Profilaggrin Demonstrated by Analysis of Tryptic Peptides†

Katheryn A. Resing,*,‡ Beverly A. Dale,‡,§ and Kenneth A. Walsh‖
Departments of Periodontics, Oral Biology, and Biochemistry, University of Washington, Seattle, Washington 98195
Received December 10, 1984

ABSTRACT: The precursor of mouse (c57/B16) epidermal filaggrin (profilaggrin) is a very large (ca. 500 000 daltons), highly phosphorylated protein containing multiple copies of filaggrin (26 000 daltons). The conversion of profilaggrin to filaggrin late in epidermal cell differentiation involves dephosphorylation and proteolysis to yield the unphosphorylated filaggrin, which polymerizes with keratin filaments into macrofibrils. In order to gain insight into the nature of these processes, we compared tryptic digests of profilaggrin with those of filaggrin by reverse-phase liquid chromatography. Approximately 80% of the profilaggrin mass consists of multiple copies of filaggrin. Twenty peptides purified in good yield from both profilaggrin and filaggrin accounted for most of the filaggrin sequence. A detailed analysis of the yield of several peptides provided an estimate of the size and frequency of the repeat unit within profilaggrin. These data indicate that the repeating substructure of profilaggrin contains about 265 amino acids and that about 50 residues are removed per filaggrin domain as the precursor is processed to filaggrin. Assuming a molecular weight of 500 000 (as estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis), this indicates there are 16 repeats. Analysis of phosphopeptides isolated from profilaggrin showed that 66% of the phosphate was located on peptides that are unphosphorylated in filaggrin. Analysis of peptide recoveries confirmed the repeat size and showed that every copy of filaggrin was phosphorylated in profilaggrin.

Epidermis is a stratified tissue with dividing basal cells that differentiate sequentially into nucleated spinous and granular cells and finally into the anucleate cells of the stratum corneum [cf. review by Odland (1983)]. This keratinization process involves reorganization of the keratin filaments of the cells. A cationic histidine-rich protein called filaggrin (also called HRP or stratum corneum basic protein, SCBP) has been isolated from rat (Dale, 1977), mouse (Steinert et al., 1981), and human (Lynley & Dale, 1983) stratum corneum and appears to interact with keratin filaments at the time of terminal differentiation (cornification) to aid in the alignment of the keratin filaments in the cornified cells (Dale et al., 1978). Filaggrin aggregates keratin filaments in vitro, forming macrofibrils with a morphology similar to that seen in the stratum corneum (Dale et al., 1978) and a stoichiometry of two filaggrin molecules for each three keratin molecules (Steinert et al., 1981).

In neonatal mouse, filaggrin has a molecular weight of 25 840 (Steinert et al., 1981) and arises in vivo from a much larger (ca. 500 000 daltons), highly phosphorylated precursor

† This work was supported by U.S. Public Health Service Grants DE04660 and DE02600 (Center for Research in Oral Biology) to B.A.D., GM15731 to K.A.W., and DE07063 to K.A.R.
‡ Department of Periodontics.
§ Department of Oral Biology.
‖ Department of Biochemistry.

0006-2960/85/0424-4167$01.50/0

(profilaggrin) located in insoluble deposits called keratohyaline granules in the granular cells (Ball et al., 1978; Scott et al., 1981; Ramsden et al., 1983; Lonsdale-Eccles et al., 1983). Phosphorylation occurs on serines (Lonsdane-Eccles et al., 1982), possibly as a result of the action of casein kinase II (Mamrack et al., 1984). Posttranslational processing of neonatal mouse profilaggrin involves dephosphorylation and proteolysis via nonphosphorylated intermediates that appear to contain two and three domains of filaggrin (Resing et al., 1984). The results of limited proteolysis suggested there are many domains of filaggrin in profilaggrin from C57/b16 mice (Lonsdale-Eccles et al., 1984). In vitro translation experiments indicated that profilaggrin is large enough to contain at least 10 domains of filaggrin (Meek et al., 1983). To test this multidomain hypothesis of profilaggrin structure and to obtain more information about the phosphorylation sites, the tryptic peptides of filaggrin and profilaggrin were examined.

Materials and Methods

Purification of Filaggrin and Its Precursors. Epidermis was obtained by a modification of a method of Mufson et al. (1977). Newborn C57/b16 mice (0–2 days) were killed by cervical dislocation, heated in a microwave oven 20 s, and frozen in powdered dry ice. After the mice were thawed, the epidermis could be peeled from the entire animal with no release of blood. The skins were kept on ice until they were homogenized in ice–cold 1.2 M NaSCN, 10 mM ethylenediaminetetraacetic acid (EDTA),[1] 3 mM *o*-phenanthroline, and 50 mM Hepes, pH 6.8, to which 0.1 mg of crystalline phenylmethanesulfonyl fluoride was added for each 10 mL of buffer. Insoluble material was removed by centrifugation, and the filaggrin-related proteins were precipitated by dilution with 10 volumes of ice-cold H_2O (Resing et al., 1984). The pellet was redissolved in 50% formic, diluted to 10%, and insoluble material removed by centrifugation at 12000*g* for 15 min at 15 °C. The pellet was reextracted in the same manner, and the extracts were lyophilized. After being redissolved in 10% formic acid, insoluble material was removed by centrifugation for 20 min in an Eppendorf tabletop centrifuge. The supernatant was fractionated on a Bio-Gel P-300 column (Bio-Rad, 121 cm × 1.5 cm) in 10% formic acid, pH 2 (NaOH), at 1.5–2 mL/h at room temperature. Fractions were analyzed on SDS–PAGE by the method of Laemmli (1970) by lyophilizing 20 μL, adding 50 μL of Laemmli's sample buffer made with Tris base, and adjusting the pH with Tris base if necessary. The profilaggrin was further purified by freezing the pooled P-300 fractions and lyophilizing for 1 h to reduce the formate concentration. This precipitated most of the profilaggrin, which was recovered by centrifugation at 12000*g* for 15 min at 0 °C. If the volume was reduced more than 30%, distilled H_2O was added before centrifugation to bring the volume to 70% of that before lyophilization.

In some cases additional purification was done by ion exchange on an SP-Sephadex column or by HPLC on an SP-TSK (Waters) column. Pooled fractions were dialyzed against 50 mM sodium formate, pH 5, applied to the column equilibrated in the same buffer and eluted by analyzing gradients made up to 1 M NaCl and 50 mM sodium formate, pH 5. Fractions were analyzed by SDS–PAGE and pooled fractions dialyzed against 10% formic and lyophilized.

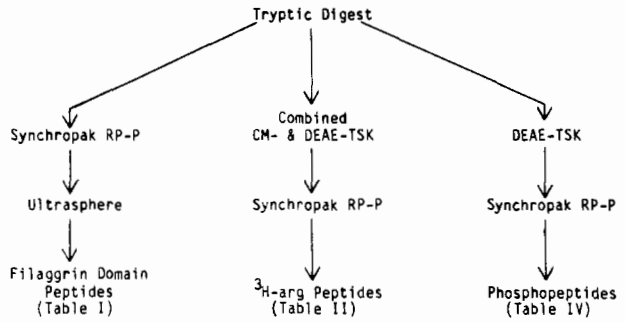

FIGURE 1: Flow diagram of purification protocol for profilaggrin and filaggrin peptides of Tables I, II, and IV. Tryptic digests and chromatographic details are described under Materials and Methods.

Radiolabeled proteins were made by injecting 50 μCi of sodium [^{32}P]phosphate or 15 μCi of [^{3}H]arginine (42 Ci/mol) intradermally into the back of newborn mice. Mice were returned to their mothers for either 8 (^{32}P) or 16 (^{3}H) h, and the protein was purified as above.

Peptide Mapping and Purification. Precipitated precursor or lyophilized filaggrin was digested with trypsin (1 mg of trypsin/50 mg of substrate in 100 mM Tris, pH 8, at 38 °C for 3 h. This peptide mixture was fractionated either by reverse-phase chromatography or ion exchange followed by reverse phase (Figure 1). For the reverse-phase system, the mixture was acidified by adding phosphoric acid to pH 2.5 and separated by HPLC on a Synchropak RP-P (Synchrom) column equilibrated in 1% TFA. Gradients were made into acetonitrile containing 0.08% TFA, with a flow rate of 2 mL/min. The effluent was monitored at 206 nm, and fractions were collected by hand. ^{32}P-Labeled samples were monitored by Cherenkov counting (without adding scintillant). Some fractions were lyophilized and dissolved in 0.1% TFA for rechromatography on an Ultrasphere column (Beckman) with a similar gradient and flow rate of 1.5 mL/min.

For ion-exchange chromatography of [^{3}H]arginine-labeled peptides, digests were first diluted with 5 volumes of water and adjusted to pH 5 with phosphoric acid. Ion-exchange HPLC used two columns (CM-TSK and DEAE-TSK, Toya-Soda) arranged in series, equilibrated in 20 nM sodium phosphate, pH 6, and eluted by applying a gradient up to 1 M NaCl and 30 nM sodium phosphate, pH 6. The initial fractionation by ion exchange was followed by reverse-phase chromatography of pooled fractions on a Synchropak RP-P column followed by an Ultrasphere column as necessary. By use of reverse-phase chromatography solvents which were volatile (Mahoney & Hermodson et al., 1980), the peptides were simultaneously desalted. To resolve phosphopeptides by ion-exchange chromatography, the peptide digest was diluted with 5 volumes of water, adjusted to pH 6 with HCl, loaded onto DEAE-TSK equilibrated in 10 mM sodium acetate, pH 6, and eluted by applying a gradient up to 0.6 M sodium phosphate and 10 mM sodium acetate, pH 6, at a flow rate of 1 mL/min. The fractions were then adjusted to pH 2.5 with 1 M phosphoric acid and further purified on a Synchropak RP-P column as above.

Characterization of Peptides and Proteins. Samples for amino acid analyses were subjected to hydrolysis for 18 h at 110 °C in 6 M HCl to which norleucine had been added as an internal standard. Hydrolyzates were then separated on a Dionex D-550 analyzer. Sequence analysis followed the automated procedure of Brauer et al. (1975) on a Beckman 890 C sequencer, or the manual method of Tarr (1982). In some cases peptides were subdigested with *Staphylococcus*

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; CM, carboxymethyl; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-*N*′-2-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton.

aureus V8 protease (1 μg/nmol of peptide) in 50 mM potassium phosphate at 35 °C for 12 h and separated on an ODS-120T column (Toya-soda), which is an end-capped column similar to the Ultrasphere column.

Results

Purification of Proteins. The thiocyanate extraction procedure described under Materials and Methods simplifies the purification of filagrin-related proteins, because the keratins are not extracted (Resing et al., 1984). In the subsequent step, all of the filaggrin-related proteins precipitate upon dilution into water, thus concentrating and partially purifying them. Subsequent extraction into 10% formic acid leaves behind contaminating nucleic acids. Apparently these contributed to the earlier precipitation, because pure filaggrin is soluble in dilute buffer.

The various filaggrin precursors and filaggrin were then resolved by gel filtration on a column of Bio-Gel P-300, as previously (Resing et al., 1984). Pooled fractions containing profilaggrin were frozen, briefly lyophilized to remove excess formic acid, and thawed on ice. The precipitate that formed was judged to be pure profilaggrin on the basis that further chromatography on DEAE-Sepharose did not change the composition. Filaggrin-containing fractions were chromatographed on SP-Sephadex or by HPLC on SP-TSK. Filaggrin was then dialyzed against 10% formic and lyophilized. Epidermis from 20 animals yielded 610 μg of filaggrin and 370 μg of profilaggrin.

Peptide Mapping and Purification Using HPLC. Three protocols for purifying peptides were used in this study (Figure 1). Initially, tryptic digests of each protein were separately fractionated on Synchropak RP-P (Figure 2). Qualitative comparison of these peptide profiles indicates that these proteins have extensive similarities, especially in the peaks eluting in the first 20 min, despite the large difference in molecular weight of filaggrin and profilaggrin. This suggested that a structural analysis of profilaggrin would be much less difficult than originally anticipated for a protein of ca. 500 000 daltons. In addition the HPLC maps showed the tryptic digest of profilaggrin could be readily resolved into less than 20 major peaks as expected if profilaggrin has a repeating substructure. A more detailed analysis was then performed to test whether the postulated multiple domains of profilaggrin each give rise to identical filaggrin molecules. In theory, nonidentity would become apparent in low yields and heterogeneity among the tryptic peptides of filaggrin, and in low molar yields of tryptic peptides from the large precursor.

It is useful to consider the profilaggrin digest as four groups of peptides, as indicated by letters A–D in Figure 2 (panel II). Rechromatography of Synchropak fractions on Ultrasphere columns (not shown) and amino acid analysis of the separated products demonstrated that most of the peptides of groups A–C were identical in both filaggrin and profilaggrin. The amino acid ratios of most of the peptides had approached integral values, and the peptides appeared to be pure. Identity of several of these peptides was confirmed by sequence analysis (Figure 3). The smaller peptides of groups A and B (peptides F1–F16 in filaggrin) were recovered in high yields, suggesting that each peptide occurs once in each copy of filaggrin. In contrast, with the exception of the large peak (labeled F17 or P17) at 17 min, peptides from group C were generally recovered in low yield from both proteins and appear to represent heterogeneous regions. Group D includes several tyrosine-containing peptides unique to profilaggrin; however, the apparent differences among them are mostly due to variable phosphorylation, as will be discussed later.

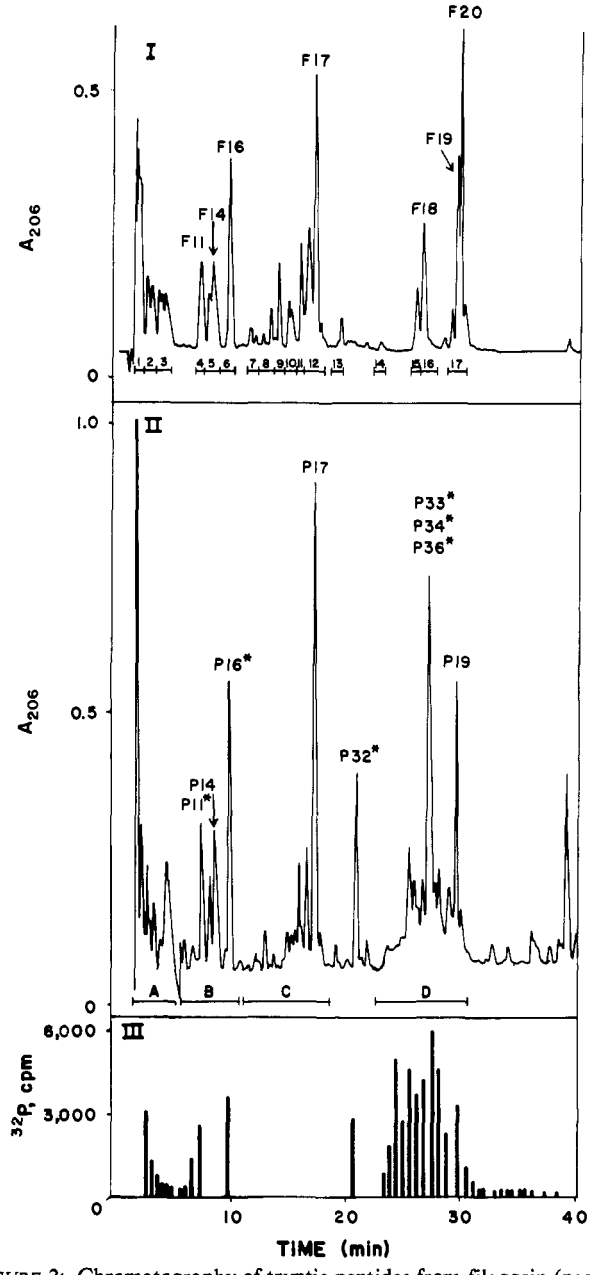

FIGURE 2: Chromatography of tryptic peptides from filaggrin (panel I) and profilaggrin (panel II) on a Synchropak RP-P column. Panel III illustrates the ^{32}P label in the profilaggrin digest (counted by Cherenkov radiation). The flow rate was 2 mL/min with a gradient from 0.1% TFA in H_2O into 0.08% TFA in acetonitrile. Filaggrin peptides referred to in the [^{3}H]arginine and ^{32}P preparations are identified by the prefix F. Analogous peptides in profilaggrin have the prefix P. Phosphorylated peptides are identified by an asterisk. Zones A–D in panel II refer to groups of peptides discussed under Results. The 17 pools in panel I were the source of the peptides in Table I.

Of the peptides recovered from both filaggrin and profilaggrin 20 were recovered from filaggrin in yields of 45% or greater (Table I). They accounted for approximately 184 of the 219 residues calculated from the molecular weight and composition and for 23 of the 24–25 arginines (calculated from the 11.9 mol % arginine in filaggrin). That these peptides account for most of the mass of filaggrin could be confirmed by comparing the integrated absorbance of peptides with that of the total absorbance profile. Since the absorbance at 206 nm reflects primarily the peptide backbone (filaggrin having no phenylalanine, tryptophan, or tyrosine), the area under individual peaks on under the total profile should reflect the peptide mass. Such an analysis showed that the purified

Table I: Tryptic Peptides Recovered from Filaggrin[a]

peptide	Synchropak pool[b]	Asx	Ser	Glx	Pro	Gly	Ala	Val	Leu	Phe	His	Arg	% yield[c]
F1	1											1.0[d]	254
F2	1		0.7									1.0	83
F3	1		1.7									1.0	68
F4	1			1.9							1.0	1.1	64
F5	1		2.3									1.1	79
F6	2	0.9	1.9									1.1	65
F7	2		0.9				1.1	0.9				1.0	58
F8	3	0.9	3.0	1.0		2.0					0.9	0.9	50
F9	3		0.9	1.9		2.0	1.0					1.0	63
F10	4	1.0			0.9							2.2	76
F11	4	0.9	1.7			1.2	1.1	0.9				0.9	51
F12	5	1.0	1.0	1.0	1.0		1.0				0.7	1.0	84
F13	5		0.9	3.0		3.1	1.9					1.0	66
F14	6			4.0		3.1	1.0				2.0	1.0	82
F15	6		0.7	3.2		1.9	1.9					0.9	58
F16	6	0.9	1.7	1.0	1.0	2.0	1.0					1.0	81
F17	12		0.8	6.8	0.9	1.8					6.2	1.0	56
F18	16	1.0	5.1	4.8	1.9	2.0		3.0			1.0	1.0	45
F19	17	1.0	5.0	2.0		6.0	6.2	1.0	1.0			1.1	52
F20	17	1.9	4.8	4.8		4.3	3.1	2.0		1.1	2.0	1.0	62
total[e]		10	36	36	6	29	19	9	1	1	14	23[f]	
expected[g]		14	44	45	7	36	19	9	1	1	18	25	

[a] Major peptides (yields >45%) from filaggrin, isolated by successive chromatography on Synchropak and Ultrasphere columns. Peptides of identical compositions were isolated from profilaggrin (some are shown in Table IV) and are identified by the prefix P instead of F. In four cases (F11, F16, F18, and F20), phosphopeptides of otherwise identical composition were found in profilaggrin. These were not phosphorylated in filaggrin. The mobility and heterogeneity of the phosphorylated forms are given in Table IV. [b] Pooled fraction source in Figure 2, panel I. Peptides were then purified by rechromatography on an Ultrasphere or Synchropak column. [c] Percent recovery from digest of filaggrin, assuming filaggrin M_r 25 840. [d] Assumed to be free arginine. [e] Nearest integer. The sum of all of the residues is 184. [f] Assuming the 254% yield of F1 indicates three separate residues of free arginine. [g] Expected number of residues, based on the composition of filaggrin and its moleculear weight of 25 840. These figures total 219 residues.

[^{3}H]-arginine labeled peptides

F14 G H Q G A H Q E Q G R

P14 G H Q G A H Q E Q G R

F17 blocked (S,Z_6,P,G_2,H_6,R)

P17 G H Q H Q H q H (S,Z_3,P,G,H_2,R)

F19 A G S S S G S G V Q G A S A G G L A A D A S R

P19 A G S S S G S G V Q G A S A G G L A A D A S R

Phosphopeptides from profilaggrin, and their filaggrin analogues

F11 V G S S A D R

P11* V G - S A - -

P11 V G S S A D R

F16 G Q S P D A S G R

P16* G Q S P D A S G R

F18 Q P S P S Q S S D S Q V H S G V Q V E G R

P18* Q P S P S Q S s d S Q v H S G v Q v e G r

F20 G V S E S Q A s e d (B,S_3,Z_2,G_2,F,H)(Z,G_3,A_2,V,H,R)

P20* G V S E s s A - - - (B,S_3,Z_2,G_2,F,H)(Z,G_3,A_2,V,H,R)

FIGURE 3: Sequence analysis of profilaggrin peptides and their filaggrin analogues. Peptides isolated from the experiments summarized in Tables I or IV were subjected to Edman degradation either in a spinning cup or by a manual method as described under Materials and Methods. F17 was blocked, although <2% had the amino terminal sequence GH; its composition was identical with that of P17, which was *not* blocked and had the partial sequence illustrated. In the case of P20* and F20, the sequence was not extended to the carboxyl-terminal arginine, but the compositions of the unsequenced region and of subpeptides generated with *S. aureus* V8 protease are shown.

peptides account for 75% of the peptide mass in the chromatogram, in rough agreement with the estimated recovery of 184 of the 219 residues. These calculations imply that the major portion of purified filaggrin has been recovered in identified peptides and is not heterogeneous.

These 20 peptides were also found in profilaggrin, but four are among phosphopeptides to be discussed later. Only a few peptides from profilaggrin could not be identified with a corresponding peptide in filaggrin (e.g. those seen at 21, 27, and 39 min in Figure 2, panel II). Thus, there is a pervasive similarity of the peptide maps of profilaggrin and filaggrin, supporting the multidomain model for profilaggrin. In seeking ways to quantitate the similarity, two approaches were used: one involved analysis of the recovery of peptides from both filaggrin and profilaggrin, and the other involved analysis of the distribution of peptides on chromatographic charts by weighing excised peak areas.

Peptide Yields from [^{3}H]Arginine-Labeled Proteins. If a peptide were present in every copy of filaggrin in a multidomain profilaggrin, then an appropriate analysis of yield of that peptide from profilaggrin should provide a count of the number of moles of that peptide per mole of profilaggrin. This necessitates correction of observed yields to absolute contents in the enzymatic digest. To accomplish this, peptides from a [^{3}H]arginine-labeled preparation were first purified, then added to an unlabeled tryptic digest of filaggrin (or profilaggrin), and repurified. The recovery of radioactivity during the repurification gave an estimate of the recovery of each such peptide during the original purification, so that its original peptide yield could be corrected to its content in the original digest (Table II). This method assumes that each peptide would be recovered in similar yield from each starting digest (whether of filaggrin or profilaggrin), a seemingly safe assumption since several preparations had almost identical yields.

For the purpose of this analysis, we chose three of the larger peptides, common to filaggrin and profilaggrin, which were

Table II: Calculation of the Size of the Repeating Unit in Profilaggrin

peptide (Table I)	yield of [^{3}H]Arg during repurification[b] (%)	filaggrin (24.5 nmol) nmol of peptide recovered	filaggrin (24.5 nmol) total nmol of peptide (corrected)[c]	profilaggrin (0.74 nmol)[a] nmol of peptide recovered	profilaggrin (0.74 nmol)[a] total nmol of peptide (corrected)[c]
F14	45	10.8	24.0	5.31	11.8
F17	54	12.5	23.2	6.09	11.3
F19	60	15.0	25.0	7.26	12.1

	filaggrin	profilaggrin
mean corrected yield (nmol)	24.1 ± 0.9	11.7 ± 0.4
nmol of peptide/nmol of protein digested	0.98	15.9
total amino acids in starting digest (nmol)[d]	5268 ± 263	3120 ± 110
nmol of amino acid residues/nmol of peptide	219 ± 13	267 ± 10

[a] By amino acid analysis, assuming a molecular weight of 500 000. [b] Pure ^{3}H peptides from filaggrin were added to an unlabeled digest, and the peptide was repurified in order to estimate the recovery during the original purification. [c] Yield of peptide after correction for losses determined by repurification of the ^{3}H peptide. [d] From amino acid analysis of an aliquot of protein before digestion.

readily purified and recovered in good yield. The three peptides chosen (Table II) contained 11, 16, and 21 amino acids, respectively, comprising 22% of the 219 residues in filaggrin. The identity of F14–P14 and F19–P19 was established by sequence analysis (Figure 3). The identity of P17 and F17 were established by composition only, since F17 was found to be blocked. During repurification of the [^{3}H]arginine-labeled filaggrin peptides (Table II), the yields were in the range 45–60%. Correction of the original yields from filaggrin indicates that 23.2–25.0 nmol of peptide was present in a digest of 24.5 nmol of filaggrin. This established the validity of the methodology, as well as showed that these three peptides were in each molecule of filaggrin.

Calculation of Size of Repeating Unit of Profilaggrin. From these data and the total amino acid content of each digest, we derived the maximum size of the filaggrin molecule and the size of the repeating unit of profilaggrin (Table II). For example, a filaggrin digest containing a total of 5268 ± 263 nmol of amino acids yielded 24.1 nmol (on the average) of each peptide. Assuming one copy of that peptide per molecule, this gives 219 ± 13 residues in filaggrin, in accord with the molecular weight of 25 840 and the average residue weight of 118. For profilaggrin, the corresponding value of the repeat unit is 267 ± 10 amino acids, or an estimated molecular weight of 31 500. The excess of 48 amino acids over filaggrin presumably represents an interdomain region that is lost during conversion of each profilaggrin repeat unit to filaggrin.

Determination of the number of repeat units in profilaggrin requires the molecular weight of the precursor, a parameter that presents special problems with such a large protein. Our current estimate of the profilaggrin mass is 500 000 daltons, based on comparing the mobility of profilaggrin on SDS–PAGE with that of a dimer of von Willebrand factor, which is estimated to have a monomer molecular mass of at least 240 000 daltons (Legaz et al., 1975). Using 500 000 as the molecular weight of profilaggrin and dividing by the estimated size of the repeat unit of 267 amino acids (31 500 daltons), we calculate that there are 15.9 ± 0.4 repeating units in profilaggrin.

Evaluation of Filaggrin Content of Profilaggrin by Gravimetric Analysis of Peak Areas. Each peptide map was analyzed for the recovery and distribution of peptide mass on the chromatogram (Table III). As noted earlier, the absorbance at 206 nm is primarily proportional to the number of peptide bonds; thus, the distribution of peptide mass in the column profile should be in proportion to the area under the profile. The areas were estimated by cutting them out of the chart paper and weighing them. Since profilaggrin must contain all of the amino acids in filaggrin, simply weighing the total profile of each protein digest and normalizing to the measured amount of a peptide present in both should give the ratio of total mass per mole of that peptide in each profile. This ratio permits calculation of the fraction of the profilaggrin mass comprising filaggrin domains.

Table III: Estimation of the Fraction of Profilaggrin That Corresponds to Filaggrin[a]

	integrated A206[b] total chromatogram (ng)	integrated A206[b] peak 19 (ng)	normalized mass, total/peak 19 (ng)	relative mass,[c] total/peak 19 (%)
profilaggrin	258.7	12.7	20.04	(100)
filaggrin	208.6	12.7	16.43	82

[a] The total mass of profilaggrin, filaggrin, or peptide 19 (P19 or F19) is recorded as the weight of the area under the absorbance profile (206 nm) of the chromatograms. [b] Chromatograms from Figure 2 were cut out and weighed as a measure of area. Error in cutting out a chromatogram was ± 5% on three repeated trials of copies of the same profile. [c] Taking profilaggrin as 100%.

The results of such an analysis are shown in Table III. These data suggest that the filaggrin domains account for 82% of the profilaggrin mass. This method assumes similar recoveries of the various peptides, an assumption that should not involve much error since the total recovery of A_{206} units from the Synchropak column is 88% of that applied. The results of this analysis are identical with those derived by quantitative reisolation of ^{3}H peptides (Table II), where 219/267 of the profilaggrin mass (82%) was calculated to be filaggrin.

Characterization of the Phosphopeptides. Examination of the phosphate distribution in the tryptic map of profilaggrin in Figure 2 (panel III) suggested that the phosphopeptides would be heterogeneous. In order to characterize these peptides, they were first purified by taking advantage of the ^{32}P label. Initial attempts using reverse-phase chromatography (Figure 2) yielded only three peptides, P11*, P16*, and P32* (at 7, 9.5, and 20 min, respectively), which were judged to be pure by amino acid analysis (Table IV) and by peak symmetry. The broad cluster of radiolabeled phosphopeptides eluting between 24 and 32 min in Figure 2 (group D) was not resolved by this method. Furthermore, lyophilization of these pooled fractions led to aggregation and nonideal behavior on reverse-phase columns. Better results were obtained by initial fractionation of the digest on a DEAE-TSK column (Figure 4) followed by rechromatography of the pooled fractions on a Synchropak column to simultaneously desalt and purify the peptides (Table IV).

About 40% of the peptide mass (estimated by A_{206}) bound to the DEAE column. Of 44 530 cpm loaded, 32 820 cpm

Table IV: Peptides Isolated from 3.66 nmol of [^{32}P]Profilaggrin[a]

phospho-peptide	non-phospho-peptide	Asx	Ser	Glx	Pro	Gly	Ala	Val	Leu	Phe	Tyr	His	Arg	sp act. (cpm/nmol)[b]	mol of PO_4^{3-}/mol of pept[c]	nmol recov[d]	% recov during repurifn[e]	cor yield (nmol)[f]	mol of pept group/mol of profilaggrin[g]	mol of pept/mol of P19
F11 analogues																				
	P11[h]	0.9	1.6			1.2	1.0	0.7					1.0			24.6				
P11*[h]		0.8	1.3			1.1	1.1	0.8					1.0		1.0	19.7				
F16 analogues																				
P16*		0.9	1.6	1.1	1.0	2.0	1.0						1.0	114	1.0	39.6	74	53.5	14.6	0.77
F18 analogues																				
P18a*		1.0	4.9	4.9	2.6	2.3		2.7				0.9	1.0	123	1.0	6.6	52			
P18b*		1.1	5.0	5.0	1.9	2.7		2.7				1.0	1.0	304	2.6	1.5				
P18c*		1.0	4.1	3.9	1.8	2.3		2.4				2.0	1.0	330	2.8	1.0				
P18d*		1.0	4.7	5.0	2.1	2.1		2.7				1.0	1.0	137	1.1	5.3	48			
P18e*		1.1	4.9	5.0	2.2	2.6		2.8				1.0	1.0	133	1.1	2.1				
P18f*		1.0	4.2	5.0	1.4	2.4		2.7				0.8	1.0	469	3.9	1.0				
	P18	1.0	4.8	5.1	1.8	2.3		2.9				1.1	1.1			12.7		60.4	16.5	0.98
																(30.2)[i]	(50)[j]			
F20 analogues																				
P20a*		1.9	4.2	5.0		3.9	2.3	1.7		0.6		2.1	1.0	141	1.1	0.9				
P20b*		1.7	4.4	5.5		4.0	2.5	2.0		1.0		2.1	1.0	158	1.3	1.3				
P20c*		1.8	4.1	5.0		4.1	2.8	1.7		1.4		2.0	1.0	142	1.1	4.0	59			
P20d*		1.7	4.0	5.0		3.9	2.5	1.7		0.8		1.9	1.1	372	3.1	1.8				
P20e*		1.9	4.5	5.1		4.1	3.0	1.9		1.1		2.1	1.2	253	2.1	13.3	71			
P20f*		1.8	4.2	5.0		4.2	2.9	2.0		0.9		1.9	1.5	386	3.2	3.6	63			
P20g*		1.9	4.1	4.7		4.0	3.0	1.9		1.0		2.0	1.1	489	4.1	11.2	67			
																(36.1)[i]	(65)[k]	55.5	15.2	0.91
P25*[h]			2.8			1.9							0.9		1.0					
Tyr-containing peptides																				
P30*		1.1	3.7	11.4		2.0					1.7	4.5	1.0	117	1.0	1.4				
P31*		1.0	3.6	10.7		4.0					2.1	4.7	0.9	108	0.9	1.6				
P32*		1.0	2.4	7.7		1.5	0.9				1.4	3.3	0.9	121	1.0	14.5	71			
P33*		1.0	3.3	12.0	0.9	3.6					3.0	5.7	0.9	139	1.1	12.9	66			
P34*		1.2	3.2	9.0		3.4					1.8	3.8	0.9	124	1.0	2.6				
P35*		1.7	4.2	11.2		3.3	0.9	1.1			1.8	4.7	0.8	373	3.1	0.4				
P36*		0.9	2.1	7.0		1.4					1.9	3.0	0.7	122	1.0	4.6	69			
P37*		1.7	4.7	10.0		5.0	1.8	1.1			1.7	4.2	1.1	521	4.3	1.4				
P38*		1.1	3.3	9.9		4.0		1.3		0.7	3.2	4.2	0.8	137	1.1	0.8				
	P39	1.2	4.1	9.0		3.9	1.1	1.1			1.7	3.8	0.9			0.6				
																(40.8)[i]	(69)[l]	59.0	16.1	0.97
	P19	0.8	5.7	1.2		6.2	5.9	0.9	1.1				1.0			51.8	85[m]	60.9	16.6	(1.0)

[a] Nomenclature: prefixes P and F define the source as profilaggrin and filaggrin, respectively. An asterisk after the peptide number identifies a phosphopeptide. Peptides P1–P20 correspond in composition to F1–F20 (Table I) and appear to represent fragments of filaggrin domains. Lower case letters in suffixes refer to chromatographically different forms of peptides with identical amino acid compositions. [b] ^{32}P was monitored by Cherenkov radiation and corrected for decay. [c] The specific radioactivity divided by 120, the approximate value for single site phosphorylation. [d] Recovery of peptide after DEAE and Synchropak chromatography (see Materials and Methods). [e] Percent of labeled peptide recovered during repurification from an unlabeled mixture as in Table II. An average percent recovery is calculated for the P18*, P20*, and tyrosine-containing groups. [f] Yield of peptide corrected for the calculated percent recovery. In each of the P18*, P20*, and tyrosine-containing groups, the average percent recovery is used. [g] A total of 1.83 mg of profilaggrin (3.66 nmol, assuming a molecular weight of 500000) was digested with trypsin. [h] P11, P25*, and P11* were not purified by the DEAE/Synchropak procedure but are included for completeness. They were isolated by successive chromatography on Synchropak and Ultrasphere columns as in Table I. The yields are adjusted to those for 1.83 mg of profilaggrin. [i] The yield of each group of peptides is summed. [j] Average of yields of P18a* and P18d*. [k] Average of yields of P20c*, P20e*, P20f*, and P20g*. [l] Average of yields of P32*, P33*, and P36*. [m] [^{3}H]Arginine-labeled P19 was added as an internal standard to an unlabeled profilaggrin digest and recovered in 85% yield during repurification. The corresponding peptide in filaggrin, F19, was found at 1 mol/mol (Table II).

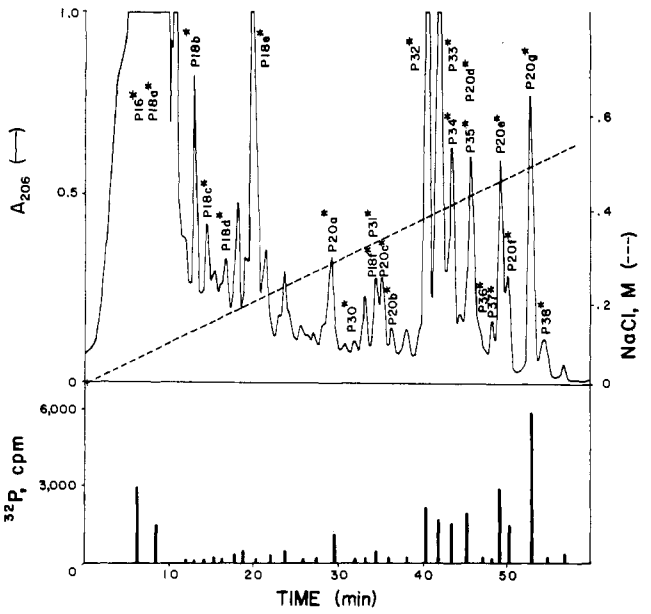

FIGURE 4: Chromatography of profilaggrin tryptic phosphopeptides on DEAE-TSK as described under Materials and Methods. The flow rate was 1 mL/min, and the gradient was 10 mM NaCl/min. Peptide numbers above the peaks indicate the pooled fractions from which that peptide was finally purified by rechromatography on a Synchropak column (Table IV). The flow-through fractions (not shown), containing P25* and P19, were collected during 10 min of loading and washing. P11* was not recovered.

(75%) were recovered after both steps of the purification. Of this, 26 525 cpm were found among the purified peptides listed in Table IV, thus accounting for 61% of the radiolabel after the two-step purification scheme. This percent recovery was sufficient to suggest that we had isolated most of the phosphopeptides, although minor components could have been missed. Indeed this purification scheme did not yield P11* at all. (It was obtained, with 6% of the total counts, in the reverse-phase experiments illustrated in Figure 2.)

Even though profilaggrin has 100–200 phosphates/mol (Lonsdale-Eccles et al., 1982; Scott & Harding, 1981), the peptides isolated by these procedures were readily divided into only six groups on the basis of similar, and in many cases identical, amino acid compositions (Table IV). For example, in the group that includes P18a*–P18f*, six phosphopeptides with different mobilities on DEAE had very similar compositions, except for a Glu/His replacement in P18c* and some variations in serine and glycine. Similarly, seven peptides fit the P20* group. Several peptides or groups of peptides had compositions identical with peptides F11, F16, F18, and F20, previously isolated from filaggrin and characterized. The identity of these filaggrin peptides with their profilaggrin-phosphorylated counterparts was confirmed by either partial or complete sequence analysis (Figure 3). The precursor form of filaggrin peptide F11 was recovered in both phosphorylated and nonphosphorylated forms (P11 and P11*). P20* and F20 showed the same amino acid terminal sequence, but sequence data could not be clearly interpreted past residue 5.

Another group of phosphopeptides from profilaggrin was characterized by their high content of glutamic/glutamine and histidine and by their content of virtually all of the tyrosine in profilaggrin. In marked contrast, no tyrosine-containing peptides were detected in a comparable digest of filaggrin (by monitoring at 277 nm its elution from a Synchropak column). These results imply that the tyrosine-containing peptides are in regions of profilaggrin that lie between the filaggrin domains.

The heterogeneity of the phosphopeptides on ion-exchange chromatography is in large part related to differences in their extent of phosphorylation. In Table IV the specific activities were normalized to 120 cpm (the approximate value for phosphorylation at a single site). The results suggest that some peptides contain a single phosphate, whereas others have two, three, or four. However, this does not explain all of the heterogeneity, since some peptides with the same composition and the same phosphate content show different elution behavior (e.g., peptides P20a*, P20b*, and P20c*). These differences may represent charge variations due to different glutamic/glutamine contents. Alternatively the pK's of the side chains of the peptide may depend upon which serine residue is phosphorylated.

Quantification of Phosphopeptide Recoveries. The absolute content of phosphopeptides was determined from the observed yields as previously described (Table II) for the nonphosphorylated peptides. But in this case peptides of identical composition were considered as a group (Table IV). As before, recoveries of 48–67% were determined by addition of a pure radiolabeled peptide to an unlabeled peptide digest and repurification of the labeled peptide. In these calculations, it was assumed that the average of the percent yield during repurification of several of the peptides was representative of the yield for all members of that peptide family. In Table IV these data are used to calculate the number of molar equivalents of peptide present in the profilaggrin digest. Peptide P19 (equivalent to F19 in Table II) was used as an internal standard to determine that the profilaggrin digest contained 60.9 nmol of filaggrin domains. As this digest began with 15 844 nmol of amino acids, the size of the repeat unit of profilaggrin could be calculated as before to be 260 amino acids.

Each phosphopeptide or phosphopeptide group was recovered in amounts comparable to that of P19, suggesting that there is one copy of each per profilaggrin repeating unit. P16* was recovered at 90% yield of that expected, possibly due to the presence of a small degree of microheterogeneity or of an unrecovered nonphosphorylated form. The sum of the yields of the peptides in the P20* group is very close to that expected if only one occurs in each domain. Peptides vary primarily in degree of phosphorylation and may also vary in amide content. If the tyrosine-containing peptides are also considered as a group, their recovery is 59 nmol out of 60.9, again implying that one such peptide is present in each profilaggrin repeat unit. Although the various P18* phosphopeptides were found with an aggregate yield only 60% of that of P19, another 40% was recovered as nonphosphorylated P18 from the breakthrough fractions of the DEAE chromatography. The presence of both phosphorylated and nonphosphorylated forms of the same peptide (P11 and P11*) was also detected in Synchropak pool 4 (Figure 2) upon rechromatography on an Ultrasphere column (data not shown).

If the corrected recovery of P19 represents the maximum number of nanomoles of repeated units, then the recovery of singly phosphorylated P16* indicates that at least 90% of this locus is phosphorylated in the whole profilaggrin molecule. Similar calculations for the P20 group indicate that at least one phosphate is present in 91% of the loci. This suggests that the various filaggrin domains are all phosphorylated to some extent, rather than that all the phosphate is in a few copies of filaggrin.

Using these data, we can calculate the average extent of phosphorylation of the profilaggrin repeat unit. For example, the P18* group of peptides has 25.4 mol of phosphate dis-

tributed among 30.2 mol of peptide, or approximately 0.8 phosphate per peptide, but varying from zero to four phosphates per peptide. The P20* group has 94 mol of phosphate/36.1 mol of peptide or an average of 2.6 phosphates per peptide. By this measure, P16* has 1 phosphate and P11* has 0.5 phosphate per filaggrin domain. Together the various forms of P11, P16, P18, and P20 contribute an average of about five phosphates per filaggrin domain.

Two other sites of phosphorylation (P25* and the P30*–P39* group) appear to be in interdomain regions, because they have no counterpart in filaggrin. Thus, each repeat unit of profilaggrin has an average of seven phosphates per domain. Multiplying these values by 16 domains gives 112 as the approximate number of phosphates on each profilaggrin molecule. However, these calculations should not be taken to mean that there are only 112 possible loci of phosphorylation on a single profilaggrin molecule. The profilaggrin population may well be heterogeneous in its phosphate content, and our method of preparation may be selecting for (or generating) a heterogeneous population of molecules. The isolation depends in part on solubility properties and appears to be biased against both the most highly phosphorylated forms (wiich do not extract as well into 10% formic acid) and the less phosphorylated forms (which do not precipitate well upon removal of formic acid after P-300 chromatography). It is not yet clear if the heterogeneity is in part generated during homogenization by the action of contaminating phosphatases. In the preparation described, no phosphatase inhibitors were added. Work is currently under way to purify and characterize profilaggrin isolated in the presence of phosphatase inhibitors.

Discussion

Prior indications of a multidomain character for profilaggrin posed two problems, one theoretical and one experimental. In theory, it seemed unlikely that the 500-kDa profilaggrin could be converted to the 26-kDa filaggrin without either discarding large segments of precursor or generating a heterogeneous product from multiple internal domains in the precursor. The extensive similarity of the peptide maps of filaggrin and profilaggrin was, by itself, an argument for a multidomain structure of profilaggrin. However, a rigorous test of the multidomain model required either evaluation of heterogeneity in filaggrin or determination of the complete sequence of the precursor, both impractical in view of the postulated large number of slightly varying filaggrin sequences and of the large size of profilaggrin. Instead, we chose to examine the partial structure of profilaggrin and to determine the absolute yield of a group of highly conserved tryptic peptides. In this way, we established that there are 16 copies of seven different filaggrin-derived peptides within 500 kDa of the precursor. Thus, if the 500-kDa estimate is correct, there must be 16 domains of virtually identical copies of filaggrin encoded within the profilaggrin sequence.

Previous peptide maps using the Cleveland technique on SDS–PAGE (Lonsdale-Eccles et al., 1981) or a Dionex amino acid analyzer (Bernstein et al., 1980) lacked the resolution of the reverse-phase system used here but clearly pointed to a multidomain structure. We have confirmed that peptides appearing to be identical by chromatographic behavior have the same amino acid compositions and, in several cases, the same sequences. The multidomain structure was confirmed by isolation of three peptides from the filaggrin domain at a yield that was 16 times the number of moles of profilaggrin. Analysis of phosphopeptides added to this picture of profilaggrin in three ways. First, each phosphopeptide (or group of related phosphopeptides) was found once per profilaggrin repeating unit. Second, the various profilaggrin repeating units appear to be phosphorylated to different extents, but with at least one phosphate present in each domain. Third, phosphate is located on the filaggrin portion of the repeating unit as well as on interdomain, tyrosine-containing peptides excised during the transformation to filaggrin.

Two different methods provided estimates of the fraction of profilaggrin that comprises copies of filaggrin. An isotope recovery technique and an integration and normalization technique each gave values of 82%. We have determined that the size of the repeating unit in profilaggrin is about 267 amino acid residues and that of filaggrin is 219 residues. Previously we have reported that about 25% of the histidine is removed during in vivo processing (Resing et al., 1984). Similarly, Steinert et al. (1984) report that 20% of the histidine incorporated into profilaggrin in cultured cells is lost during processing to filaggrin. Since the mole percents of histidine in profilaggrin and filaggrin are nearly identical, these measurements are in accord with our present estimate that about 20% of the mass is removed.

The mature protein filaggrin shows little evidence of microheterogeneity, indicating that the various domains of profilaggrin are nearly identical with one another. This is confirmed by sequence analysis for nearly one-third of its length. However, it should be noted that one or two different copies out of 16 might have been overlooked. Some heterogeneity is indicated by the low yields of certain filaggrin-derived peptides (perhaps 25% of the peptides) and of the phosphopeptides derived from profilaggrin. Harding & Scott (1983) have demonstrated heterogeneity of charge in filaggrins using nonequilibrium isoelectric focusing. Some of this microheterogeneity may be attributed to variations in Glu/Gln and Asp/Asn as seem to occur in the phosphopeptides analyzed here.

The major difference between the peptide maps of filaggrin and profilaggrin is accounted for in profilaggrin peptides P18*, P20* and the tyrosine-containing peptides. From filaggrin we isolated only nonphosphorylated peptides F18 and F20. There were no filaggrin peptides corresponding to the tyrosine-containing peptides, and these must represent segments removed during the proteolytic excision of interdomain regions.

We can now suggest a more detailed model of the structure of profilaggrin (Figure 5). In C57/b16 neonatal mice, profilaggrin appears to be a 500-kDa protein, with 16 filaggrin domains and about 20% interdomain material ($\sim$50 amino acids) which is lost during processing. If the tyrosine-containing peptides are representative of the interdomain regions, they are more heterogeneous than the filaggrin domains themselves (Table IV). We are now accumulating evidence of sequence heterogeneity among these peptides (K. A. Resing, unpublished observations). The estimate of 50 amino acids represents an average value for the interdomain regions, but we have little evidence of any variation in length. Limited proteolysis on SDS–PAGE does suggest that the repeat units are similar in size (Lonsdale-Eccles et al., 1984). Since filaggrin itself is blocked (Lonsdale-Eccles et al., 1982), and so is its tryptic peptide F17, one can suggest that this peptide represents the amino terminus of filaggrin that has become blocked in vivo and is perhaps the last processing step in the conversion of profilaggrin to filaggrin. The equivalent peptide (P17) was generated in unblocked form by tryptic digestion of profilaggrin, in accord with this hypothesis.

The unusually large number of repeating units in profilaggrin has few precedents in nature. Precursors of enkephalins, yeast mating factor, epidermal growth factor, and

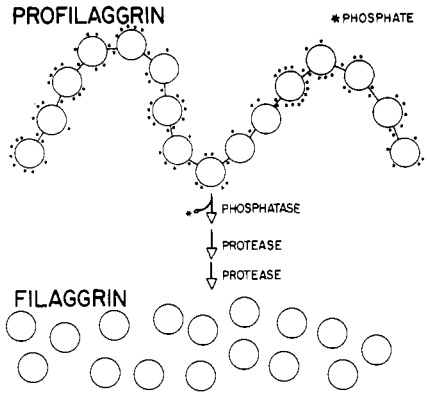

FIGURE 5: Schematic diagram of the structure of profilaggrin. The filaggrin domains (represented by circles) are connected by interdomain regions straight lines) 50 amino acids long. Phosphates are indicated by asterisks with an average of five phosphates per domain and a random distribution among the domains. In the process of cornification, profilaggrin is dephosphorylated, and filaggrin is released via at least two distinct proteolysis steps (Resing et al., 1984).

ubiquitin undergo similar proteolytic processing of identical, or nearly identical, segments from a much larger precursor (Comb et al., 1982; Kurjan & Kerskiowitz, 1982; Ullrich et al., 1983; Dworkin-Rastl et al., 1984), but in no case has there been reported such a long repeated domain. Although profilaggrin probably originated by tandem gene duplication, the high degree of similarity maintained among the copies suggests that gene conversion may be involved in maintaining that similarity (Klein & Petes, 1981).

By expanding our knowledge of the structure of profilaggrin, we are now able to direct our efforts toward characterization of the posttranslational steps involved in the generation of filaggrin. For example, it has been suggested that the kinase involved in phosphorylation of profilaggrin may be similar in specificity to casein kinase II. All of the phosphopeptides sequenced have an acidic residue located in the proper position to satisfy the specificity requirements of casein kinase II (Pinna et al., 1979). Mamrack et al. (1984) have reported phosphorylation of filaggrin in vitro by epidermal casein kinase II. Our tryptic peptides P18* and P20* have multiple phosphorylation sites, another characteristic of casein kinase II substrates (Mercier, 1981). Finally it can be surmised that the phosphorylation of profilaggrin would induce major changes in the nature of its filaggrin domains by adding ~7- charges at pH 7. These phosphoryl groups may protect profilaggrin from premature proteolysis to filaggrin. Lonsdale-Eccles et al. (1982) showed that a 52-kDa, phosphorylated fragment of rat profilaggrin (which is now thought to contain one domain of filaggrin and an interdomain region) did not interact with keratins to form macrofibrils, in contrast to mature, nonphosphorylated filaggrin. During epidermal differentiation, dephosphorylation of profilaggrin appears to precede the generation of nonphosphorylated two (or three) domain intermediates and filaggrin (Resing et al., 1984). The intermediates are capable of interacting with keratins (Harding & Scott, 1983), suggesting that dephosphorylation of profilaggrin may be the controlling first step in the generation of filaggrin and the formation of keratin macrofibrils during differentiation.

ACKNOWLEDGMENTS

We thank Roger Wade and Lowell Ericsson for their technical assistance and Drs. Tatsuru Sasagawa, Koji Takio, and Koiti Titani for many helpful discussions concerning peptide isolations. We owe a special debt to Santosh Kumar for her advice and support.

REFERENCES

Ball, R. C., Walker, G. K., & Bernstein, I. A. (1978) *J. Biol. Chem. 253*, 5861–5868.

Bernstein, I. A., Brabec, R. K., Badlamuci, B., Ohno, A. K., Delap, L. W., Gray, R. H. (1980) in *Biochemistry of Normal and Abnormal Differentiation* (Bernstein, I. A., & Seiji, M., Eds.) pp 421–432, University of Tokyo Press, Tokyo.

Brauer, A. W., Margolies, M. N., & Haber, E. (1975) *Biochemistry 14*, 3029–3035.

Comb, M., Seeburg, P. H., Adelman, J., Eiden, L., & Herbert, E. (1982) *Nature (London) 295*, 663–666.

Dale, B. A. (1977) *Biochim. Biophys. Acta 491*, 193–204.

Dale, B. A., Holbrook, K. A., & Steinert, P. M. (1978) *Nature (London) 276*, 729–731.

Dworkin-Rastl, E., Shrutkowski, A., & Dworkin, M. B. (1984) *Cell (Cambridge, Mass.) 39*, 321–325.

Harding, C. R., & Scott, I. R. (1983) *J. Mol. Biol. 170*, 651–673.

Klein, H. L., & Petes, T. D. (1981) *Nature (London) 289*, 144–148.

Kurjan, J., & Herskowitz, I. (1982) *Cell (Cambridge, Mass.) 30*, 933–943.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lonsdale-Eccles, J. D., & Dale, B. A. (1983) *Biochem. Soc. Trans. 11*, 195–196.

Lonsdale-Eccles, J. D., Teller, D. C., & Dale, B. A. (1982) *Biochemistry 21*, 5940–5948.

Lonsdale-Eccles, J. D., Resing, K. A., Meek, R. L., & Dale, B. A. (1984) *Biochemistry 23*, 1239–1245.

Lynley, A. M., & Dale, B. A. (1983) *Biochim. Biophys. Acta 744*, 28–35.

Mahoney, W. C., & Hermodson, M. A. (1980) *J. Biol. Chem. 255*, 11199–11205.

Mamrack, M. D., Klein-Szanto, A. J. P., Reiners, J. J., & Slaga, T. J. (1984) *Cancer Res. 44*, 2634–2641.

Meek, R. L., Lonsdale-Eccles, J. D., & Dale, B. A. (1983) *Biochemistry 22*, 4867–4871.

Mercier, J.-C. (1981) *Biochimie 63*, 1–17.

Mufson, R. A., DeYoung, L. M., & Boutwell, R. K. (1977) *J. Invest. Dermatol. 69*, 547–550.

Odland, G. F. (1983) in *Biochemistry and Physiology of the Skin* (Goldsmith, L. A., Ed.) Vol. 1, pp 3–63, Oxford University Press, New York, NY.

Pinna, L. A., Donella-Deana, A., & Meggio, F. (1979) *Biochem. Biophys. Res. Commun. 87*, 114–120.

Ramsdan, M., Loehren, D., & Balmain, A. (1983) *Differentiation (Berlin) 23*, 243–249.

Resing, K. A., Walsh, K. A., & Dale, B. A. (1984) *J. Cell Biol. 99*, 1372–1378.

Scott, I. R., & Harding, C. R. (1981) *Biochim. Biophys. Acta 668*, 65–78.

Scott, I. R., & Harding, C. R. (1982) *Biochem. Soc. Trans. 9*, 1981–1982.

Steinert, P. M., Cantieri, J. S., Teller, D. C., Lonsdale-Eccles, J. D., & Dale, B. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4097–4101.

Tarr, G. E. (1982) in *Methods of Protein Sequence Analysis* (Elzinga, M., Ed.) pp 223–232, Humana Press, Clifton, NJ.

Ullrich, A., Gray, A., Berman, C., & Dull, T. J. (1983) *Nature (London) 303*, 821–825.