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Epidermal Filaggrin Is Synthesized on a Large Messenger Ribonucleic Acid as a High-Molecular-Weight Precursor†

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ABSTRACT: Filaggrin is a keratin filament associated protein found in the differentiated cells of the epidermis. The mouse protein has a molecular weight of 26.5×10^3, while the molecular weight of rat filaggrin is 49×10^3, when analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. In order to clarify our understanding of the precursor form of filaggrin, RNA was isolated from mouse and rat epidermis and the poly(A+) fraction was translated in a cell-free protein synthesis system. Immunoprecipitation of translated proteins with specific antibody against rat filaggrin revealed a diffuse radiolabeled band with a molecular weight of approximately 250×10^3 on SDS–polyacrylamide gels. The size of filaggrin mRNA was estimated by sedimenting poly(A+) RNA through isokinetic sucrose gradients. Mouse filaggrin mRNA activity was located in the 30S region of the gradient, while rat filaggrin mRNA was located in the 34S region. The molecular weight of rat filaggrin mRNA was estimated to be 5×10^6 by electrophoresis in denaturing agarose gels containing methylmercury(II) hydroxide. A messenger RNA of this size could code for a polypeptide as large as approximately 600 kilodaltons. We conclude that filaggrin is synthesized as a large molecular weight precursor that must undergo substantial processing prior to formation of the mature form of the protein.

The epidermis is a continuously differentiating tissue in which the basal cells proliferate and sequentially form spinous, granular, and finally the fully differentiated cells of the stratum corneum. These cells are filled with keratin filaments embedded in a matrix material and surrounded by the thickened "cornified cell envelope" of cross-linked protein (Brody, 1959; Rice & Green, 1977). The matrix material is thought to be the protein filaggrin, an unusually polar cationic protein, rich in histidine, that aggregates with keratin filaments to form macrofibrils (Dale et al., 1978; Dale, 1977; Steinert et al., 1981). The mechanisms that control the differentiation of epidermal cells are currently under investigation in several laboratories (Rice & Green, 1978; Hennings et al., 1980; Fuchs & Green, 1980, 1981; Roop et al., 1983; Watt & Green, 1982). In order to delineate these control mechanisms at the biochemical level, it is necessary to identify discrete biochemical markers and understand their biosynthesis.

A histidine-rich protein localized in keratohyalin granules has long been recognized as a marker of differentiation of cells of the granular layer (Fukuyama & Epstein, 1966; Hoober & Bernstein, 1966; Sibrack et al., 1974; Balmain et al., 1977; Ball et al., 1978). This histidine-rich protein is a phosphorylated precursor form of filaggrin (Dale & Ling, 1979; Lonsdale-Eccles et al., 1980). Its conversion to the nonphosphorylated filaggrin apparently occurs at the time of conversion of granular cells to the anucleate cornified cells and may serve as a differentiation marker for the appearance of cornified cells (Ball et al., 1978).

Although significant advances have been made defining physical, biochemical, and functional properties of this structural protein, efforts to elucidate its biosynthetic pathway have been severely hampered by uncertainty in the molecular weight of the precursor. The filaggrin precursor is readily degraded during extraction from the epidermis (Scott & Harding, 1981). As a result, values reported for its molecular weight have varied by nearly two orders of magnitude even within a single species (Sibrack et al., 1974; Ball et al., 1978; Dale & Ling, 1979; Murozuka et al., 1979; Lonsdale-Eccles et al., 1980, 1982; Scott & Harding, 1981).

We have addressed the question of filaggrin biosynthesis by isolating filaggrin mRNA from mouse and rat epidermis. We find that filaggrin mRNA is unusually large (30S and

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34S) and can be translated in a cell-free protein synthesis system to yield a large (probably incomplete) protein of M_r approximately 250 × 10^3. Therefore, the initial step of filaggrin biosynthesis is the translation of a large precursor molecule that must undergo substantial proteolytic processing to yield the mature form of mouse (26.5-kilodalton) and rat (49-kilodalton) filaggrin.

Materials and Methods

RNA Isolation. Epidermis from 1-day-old Balb/c mice and Sprague-Dawley rats was obtained as previously described (Lonsdale-Eccles et al., 1982). The epidermis was frozen on dry ice immediately after separation from the dermis and stored at −70 °C. The frozen tissue was homogenized on a Tissuemizer (Brinkman Instruments) for 60 s in 1% SDS,[1] 10 mM EDTA, 10 mM Tris-HCl, pH 7.5, and proteinase K (50 μg/mL) and incubated for 60 min at 55 °C. The digests were extracted twice with phenol/chloroform (1:1) and then with chloroform. The aqueous phases were made 100 mM with NaCl, and nucleic acids precipitated at −20 °C after 2 volumes of ethanol was added. The precipitate was collected by centrifugation at 15000*g* for 10 min, washed with 40 mM NaCl in 70% ethanol, and dissolved in water. RNA was precipitated with 2 M LiCl and 10 mM NaOAc, pH 5, while tRNA, 5S RNA, and DNA remained soluble (Palmiter, 1973). The yield of RNA was 3–4 mg/g (wet weight) of epidermis. Poly(A+) RNA was isolated by chromatography on oligo(dT)–cellulose (Aviv & Leder, 1972).

Cell-Free Translation and Immunoprecipitation. A rabbit reticulocyte lysate was prepared and modified (by gel filtration) as described by Palmiter et al. (1977), except that the K^+ final concentration was 160 mM. Translations to identify filaggrin mRNA activity were carried out in the presence of a mixture of ^{3}H-labeled L-arginine, L-alanine, and L-histidine at 150 μCi/mL each. Reaction mixtures were incubated for 1.5–2 h at 26 °C. Aliquots for gel electrophoresis were diluted with an equal volume of 2× sample buffer (125 mM Tris-HCl, pH 6.7, 4% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.25% bromphenol blue) and heated for 3 min at 100 °C. Aliquots for immunoprecipitation were diluted with 3 volumes of TNENN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% NP-40, and 0.1% NaN_3). Five microliters of rabbit antiserum to rat filaggrin (Dale et al., 1981) (previously diluted 1:10 in TNENN buffer) was added, and samples were incubated at 26 °C for 30 min. Ten microliters of Pansorbin (Calbiochem) was added at 0 °C. After 30 min the samples were pelleted through 400 μL of 1.0 M sucrose in TNENN buffer at 15000*g* for 10 min in 5 × 60 mM glass tubes (Kimble) in adapters fitted to an HB-4 rotor (Sorvall). After the pellets were resuspended in TNENN buffer, Pansorbin was collected by centrifugation and resuspended in 60 μL of 1× sample buffer (65 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 10% glycerol) heated at 100 °C for 3 min and the Pansorbin pelleted. The supernatants were collected and analyzed on SDS–polyacrylamide gels (Laemmli, 1970) and prepared for fluorography with Enhance (New England Nuclear). The molecular weights of marker proteins (×10^{-3}) are indicated on the figures. [^{3}H]Fibronectin was a kind gift of Dr. Paul Bornstein.

Sedimentation of RNA through Isokinetic Sucrose Gradients. Poly(A+) epidermal RNA (200 μg) in 200 μL of water was heated at 68 °C for 3 min, cooled on ice, and diluted with an equal volume of 2× buffer (20 mM Tris-HCl, pH 7.5, and 0.2 mM EDTA). The RNA was layered over a linear 5–20% sucrose gradient (prepared in the same buffer) and centrifuged at 40000*g* for 4.5 h in a SW-41 rotor (Beckman). Fractions were collected from the bottom, made 100 mM with NaCl, and precipitated with 2 volumes of ethanol at −20 °C. RNA was recovered by centrifugation; the pellets were washed with 70% EtOH, air-dried, and dissolved in water. RNA from each fraction was translated as described above. Ribosomal RNA (200 μg of rat epidermis) was applied to parallel gradients. After centrifugation the tubes were pumped from the bottom, and absorbance at 254 nm was measured with an Isco Model 160 sucrose gradient fractionator.

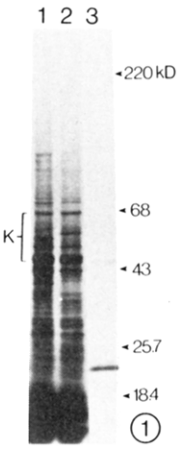

FIGURE 1: Cell-free synthesis of rat and mouse epidermal proteins. Poly(A+) epidermal RNA was translated in the presence of [^{35}S]-methionine (1000 Ci/mol, 500 μCi/mL). After 90-min incubation, the radiolabeled polypeptides were analyzed on SDS 7.5–15% gradient polyacrylamide gels. A fluorogram of the dried gel is shown. Lane 1, mRNA from rat epidermis; lane 2, mRNA from mouse epidermis; lane 3, no mRNA added. The molecular weight of marker proteins (×10^{-3}) is indicated on the right. The position of the keratins is indicated by K.

Electrophoresis of RNA in Denaturing Gels. Horizontal gels (4 mm thick) were cast from 0.85% low-melting agarose (BRL) in E buffer containing 20 mM methylmercury(II) hydroxide (Bailey & Davidson, 1976). Poly(A+) RNA (30 μg) in E buffer with methylmercury(II) hydroxide was applied to a single well 1.2 cm wide. Electrophoresis was carried out at 60 V and stopped when the bromphenol blue reached the bottom. The gel was soaked in 100 mM β-mercaptoethanol (three changes for 20 min each). Lanes with rRNA standards were removed and stained with ethidium bromide (0.5 μg/mL). The lane containing mRNA was cut into 2-mm slices, the agarose melted at 68 °C in 0.5 mL of 500 mM ammonium acetate, and the aqueous solution extracted with phenol and then twice with chloroform (Lemischka et al., 1981). Five micrograms of wheat germ tRNA was added, and the samples were precipitated with 2 volumes of ethanol. The RNA was recovered by centrifugation, washed with 70% ethanol, and dissolved in water. Translation of fractions and immunoprecipitation of filaggrin-related products were carried out as described above.

Results

Isolation and Translation of Epidermal mRNA. Epidermal RNA [poly(A+)] was translated in a cell-free rabbit reticulocyte system in the presence of [^{35}S]methionine. Analysis of the radiolabeled translation products by SDS–polyacrylamide gel electrophoresis is shown in the fluorograph of Figure 1. Translated proteins varied from those of small molecular weight (M_r 20 × 10^3) to easily detected polypeptides of M_r

[1] Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; poly(A+), poly(adenylic acid) containing.

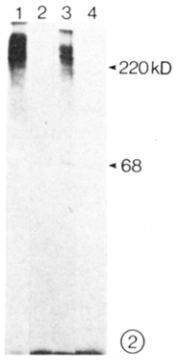

FIGURE 2: Cell-free translation of rat and mouse filaggrin polypeptides. Poly(A+) RNA enriched for filaggrin mRNA on sucrose gradients was translated in 40 μL of a protein synthesis reaction mixture containing ^{3}H-labeled alanine, arginine, and histidine (150 μCi/mL each). After a 2-h incubation, half was immunoprecipitated with antifilaggrin antiserum and the other half with preimmune serum (as described under Materials and Methods). Immunoreactive products were analyzed on 7% SDS–polyacrylamide gels. Immunoprecipitated filaggrin translation products were from rat (lane 1) and mouse (lane 3) epidermal mRNA. Control precipitations were with preimmune serum of translations using rat (lane 2) and mouse (lane 4) epidermal mRNA translations.

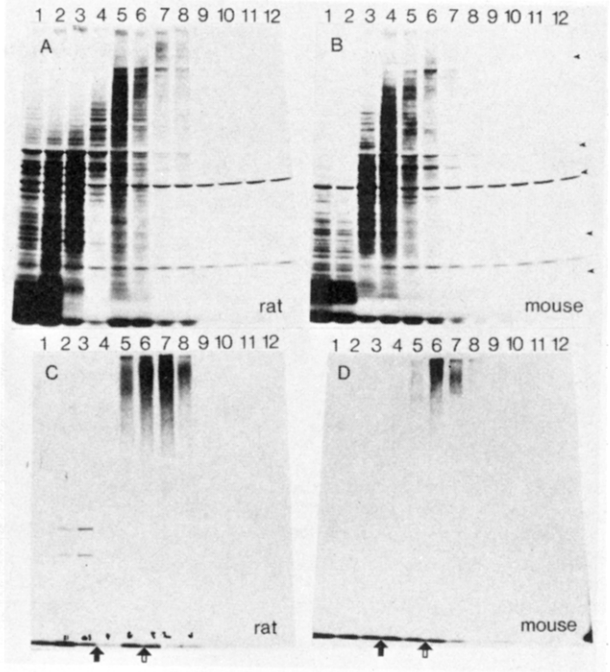

FIGURE 3: Fractionation of epidermal mRNA on isokinetic sucrose gradients and identification of filaggrin mRNA activity. Epidermal poly(A+) RNA from rat (A and C) and mouse (B and D) was sedimented through isokinetic sucrose gradients (5–20%) as described under Materials and Methods. An aliquot of each fraction was translated in the presence of [^{35}S]methionine (500 μCi/mL) for 90 min (A and B), and products were analyzed on SDS 7.5–15% gradient polyacrylamide gels. A second aliquot of each fraction was translated in the presence of ^{3}H-labeled alanine, arginine, and histidine (C and D), then immunoprecipitated, and analyzed on SDS–polyacrylamide gels (7%). The position of the marker proteins (as in Figure 1) is indicated on the right. The direction of sedimentation is from left to right. The radiolabeled proteins in fractions 2 and 3 of (C) are also present in preimmune control precipitations of poly(A+) mRNA translations (not shown). Epidermal ribosomal RNA (200 μg) was sedimented in parallel tubes. The gradients were fractionated, and the absorbance at 254 nm was monitored in a flow cell. The position of the 18S (closed-tailed arrow) and 28S (open-tailed arrow) peaks is shown. *s* values for the filaggrin mRNAs were calculated relative to these peaks as indicated in the text.

approximately 200 × 10^3. Several major proteins are present with molecular weights ranging from 40 × 10^3 to 70 × 10^3. These are most likely epidermal keratins and actin.

Filaggrin does not possess either of the sulfur-containing amino acids, methionine or cysteine; however, alanine, arginine, and histidine are abundant (Dale, 1977; Steinert et al., 1981). Therefore, all translations to identify filaggrin were carried out in the presence of ^{3}H-labeled alanine, arginine, and histidine, followed by immunoprecipitation with antibodies to rat filaggrin and analysis on SDS–polyacrylamide gels. Initial experiments revealed specifically immunoprecipitated translation products that did not enter a 10% acrylamide gel. However, no immunoprecipitated polypeptides migrated with molecular weights in the range of the mouse or rat filaggrin (data not shown). Analysis on 7% or 4–12% gradient gels revealed that translated filaggrin-related polypeptides appeared as a diffuse band with an apparent molecular weight of 250 × 10^3; these were most readily detected with sucrose gradient enriched poly(A+) RNA (Figure 2). Lesser amounts of filaggrin-related polypeptides were also observed as a smear on the gel between 250 and 100 kilodaltons.

One possible explanation for the diffuse nature of the immunoprecipitated band is that the nascent chains were aggregated. Therefore, we tried a variety of modifications to eliminate possible aggregation artifacts and to improve the mobility of the filaggrin translation products on SDS gels. The samples were heated at 100 °C for 60 min, the SDS concentration in the sample buffer and in the electrophoresis buffer was doubled, analysis was carried out on SDS gels containing 6 M urea, samples were not heated above 15 °C, and immunoprecipitation was performed with protein A coupled Sepharose instead of Pansorbin. The basic pattern of ^{3}H-labeled filaggrin polypeptides was not altered by any of these procedures.

In addition, a time-course study of filaggrin polypeptide synthesis was performed. Aliquots were removed at various times during the translation of epidermal poly(A+) RNA, and translation was stopped with cycloheximide. Filaggrin translation products were immunoprecipitated and analyzed on SDS–polyacrylamide gels. After 20 min filaggrin polypeptides were as large as M_r 40 × 10^3, at 40 min M_r 100 × 10^3, and at 90 min M_r 200 × 10^3 (data not shown). Thus the high-molecular-weight filaggrin polypeptides are not an aggregation artifact but appear in a time-dependent manner during translation. We conclude that the smear of filaggrin polypeptides translated in our cell-free system is most likely a result of incomplete synthesis of a very large filaggrin protein (M_r >250 × 10^3).

Size of the Filaggrin mRNA. Poly(A+) RNA from both mouse and rat epidermis was sedimented through isokinetic sucrose gradients to determine the approximate size of the filaggrin mRNA and to determine if it is large enough to code for the large proteins identified in Figure 2. After fractionation of the gradients and recovery of the mRNA, cell-free translations were carried out in the presence of [^{35}S]methionine (Figure 3A,B). Because the centrifugation time was short, separation of various-sized mRNA molecules was not extensive. The largest, readily detectable mRNA coded for a [^{35}S]Met-labeled protein of approximately 185 kilodaltons and only migrated two-thirds of the distance of the gradient, fractions 7 and 8. Filaggrin mRNA activity, determined by cell-free translation in the presence of tritiated amino acids and immunoprecipitation as in Figure 2, is shown in Figure 3C,D. Each lane of an appropriately exposed fluorograph was scanned with a microdensitometer, and the peak areas were determined. Mouse and rat filaggrin mRNA activity appears in the 30S and 34S region of the gradient, respectively, relative

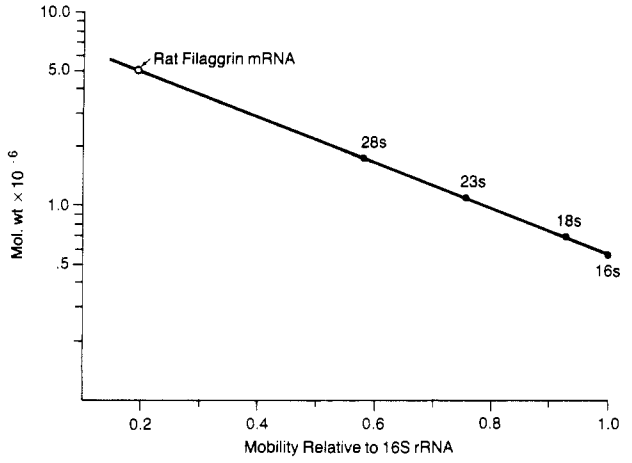

FIGURE 4: Identification and molecular weight determination of rat filaggrin mRNA after electrophoresis through denaturing agarose gels. RNA was separated on 0.85% agarose containing 20 mM CH_3Hg, the lane sliced, and RNA eluted, as described under Materials and Methods. Fifty percent of the RNA recovered from each gel slice was translated in 20 μL of translation reaction mixture containing a mixture of ^{3}H-labeled alanine, arginine, and histidine, immunoprecipitated, and analyzed on 4–12% SDS–polyacrylamide gels. The mobility of mRNA that translated filaggrin immunoprecipitable product is shown relative to the mobility of the 16S rRNA standard. The positions of 16S, 18S, 23S, and 28S rRNAs are also indicated. The following molecular weights have been assigned to rRNA according to Wellauer et al. (1974) and Attardi & Amaldi (1970): 28S, 1.75×10^6; 23S, 1.1×10^6; 18S, 0.68×10^6; 16S, 0.55×10^6. The calculated value for rat filaggrin mRNA is 5×10^6 daltons.

to the migration of 18S and 28S ribosomal RNA. These data show not only that filaggrin mRNA is large but also that rat and mouse mRNA are a different size.

In order to obtain a more accurate molecular weight of filaggrin mRNA, rat epidermal RNA was separated by electrophoresis under fully denaturing conditions through 0.85% agarose gels containing 20 mM methylmercury. Lanes containing mRNA were sliced, and the mRNA was eluted and then translated. The radiolabeled immunoprecipitated proteins were analyzed by SDS–polyacrylamide gel electrophoresis. Filaggrin mRNA activity appeared as a single peak, with a molecular weight estimated to be 5×10^6 by extrapolation of a standard curve plotted from the migration of rRNA markers (Figure 4). An mRNA of this size would code for a polypeptide of approximately 600 kilodaltons if all of the mRNA was a coding sequence.

Discussion

The most direct way to evaluate the size of the filaggrin precursor is to isolate the messenger RNA and translate it in a cell-free protein synthesizing system. In the work presented here, we demonstrate that rat filaggrin mRNA is large (34S, 5×10^6 daltons) and theoretically contains information to code for a protein of 600 kilodaltons. Indeed, we have now identified mouse and rat phosphorylated filaggrin precursor with molecular weights of 390×10^3 and 600×10^3, respectively (J. D. Lonsdale-Eccles, K. A. Resing, R. L. Meek, and B. A. Dale, unpublished experiments). High-molecular-weight proteins rapidly labeled with [^{3}H]histidine have also been identified by others (Scott & Harding, 1981; Ramsden et al., 1983).

Along with fibroin mRNA (M_r 5–6 × 10^6; Lizardi, 1979) and thyroglobulin mRNA (M_r 2.8 × 10^6; Vassart et al., 1977), filaggrin mRNA is one of the largest isolated from eukaryotic tissues. Mouse filaggrin mRNA is also large (30S), yet not as large as that of rat (34S); the size difference between the species is also reflected in the filaggrin precursors (J. D. Lonsdale-Eccles et al., unpublished experiments).

Translation of filaggrin mRNA in a cell-free system yields immunoprecipitable incomplete filaggrin polypeptides as large as 250 kilodaltons. This work, along with that of others (J. D. Lonsdale-Eccles et al., unpublished experiments; Ramsden et al., 1983), provides strong evidence that filaggrin is synthesized as a large precursor. Two possible models for the filaggrin precursor structure are envisaged: (1) functional filaggrin is only 5–10% of the precursor and the remaining portion is nonfilaggrin sequences, or (2) filaggrin sequences are repeated many times in the precursor. We suggest the second possibility. Filaggrin itself lacks the amino acid methionine but is rich in histidine, arginine, serine, and alanine. The large, immunoprecipitable, cell-free translation product could not be labeled with [^{35}S]methionine but was labeled with a mixture of tritiated amino acids, consistent with the composition of the filaggrin itself. Methionine labeling would be expected if 90% of the translation product was random nonfilaggrin amino acid sequences. Moreover, recent peptide mapping studies of the large precursor extracted from tissue show that few, if any, precursor peptides are unrelated to those of filaggrin (J. D. Lonsdale-Eccles et al., unpublished experiments). We propose that the filaggrin precursor is composed of tandemly linked domains of filaggrin. This type of repeated structure is somewhat similar to the precursors of enkephalins (Herbert & Uhler, 1982) and tandem repeats of yeast α-mating factor (Kurjan & Herskowitz, 1982). Posttranslational proteolysis is required in each case to obtain the functional products.

The isolation of the filaggrin mRNA and its translation in vitro into a protein much larger than filaggrin itself support the previous findings of a large, rapidly labeled, histidine-rich protein precursor of filaggrin (Ball et al., 1978; Scott & Harding, 1981). The large nascent precursor must undergo substantial posttranslational processing to yield the final filaggrin proteins of 26.5 and 49 kilodaltons in the mouse and rat, respectively. This processing includes phosphorylation via the action of one or more kinases to form the highly phosphorylated precursor (Lonsdale-Eccles et al., 1980), followed by protease and/or phosphatase action to yield the mature protein. The isolation of the filaggrin mRNA will permit studies on these posttranslational modifications of the nascent precursor chain. The large size of the mRNA has already led us to propose a new model for the filaggrin precursor structure (J. D. Lonsdale-Eccles et al., unpublished results). Preparation and cloning of the cDNA will permit testing of the tandem-repeating domain structure of the filaggrin precursor and allow detailed analysis of filaggrin gene expression during epidermal differentiation.

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