Alteration in the Distribution of the Epidermal Protein Filaggrin during Two-Stage Chemical Carcinogenesis in the SENCAR Mouse Skin

M. D. Mamrack, A. J. P. Klein-Szanto, J. J. Reiners, Jr., and T. J. Slaga

ABSTRACT

The histidine-rich epidermal protein filaggrin was purified from urea extracts of newborn SENCAR mouse epidermis. The protein had a molecular weight of 28,000 and an amino acid composition distinctive for this class of proteins. The purified protein was a phosphate acceptor in an in vitro protein kinase assay. Rabbit antibodies raised against filaggrin were used in an indirect immunofluorescent survey of the distribution of filaggrin in the epidermis after single and multiple 12-O-tetradecanoylphorbol-13-acetate treatments as well as in papillomas and carcinomas. The immunofluorescent pattern of acetone-treated adult SENCAR mouse epidermis showed primarily granular layer fluorescence. A single topical 12-O-tetradecanoylphorbol-13-acetate treatment increased immunofluorescence in basal and suprabasal cells. Large papillomas produced by a dimethylbenz[a]anthracene initiation-12-O-tetradecanoylphorbol-13-acetate promotion protocol showed increased fluorescence in all layers. Exuberant papillomas showed a pleomorphic distribution of filagrin with alternating positive and negative areas of immunofluorescence. Filaggrin immunofluorescence in invasive carcinomas was negative or only slightly positive. The distribution of filagrin as detected by indirect immunofluorescence is a good indicator of maturation and differentiation in experimental tumors, and its presence correlates with the absence of aggressive or invasive growth.

INTRODUCTION

The name filaggrin has been proposed for the histidine-rich basic protein that was originally isolated from the stratum corneum of rat or mouse epidermis by a number of investigators (1, 3, 11, 36). It was reported that this protein was associated with keratin filaments in the stratum corneum and that a highly phosphorylated, higher-molecular-weight form of filaggrin was localized in keratohyalin granules (1, 3, 8, 9, 15, 22, 34). Whether filaggrin is synthesized as a high-molecular-weight precursor is a question still in question, as is its role in the formation of keratohyalin granules (4, 22, 34). It has been observed that dephosphorylated filaggrin is capable of forming macrofilaments in vitro when mixed with intermediate filaments consisting of keratin, desmin, or decamin (7, 36). This property of aggregating various intermediate filaments extends the original finding that the release of active filaggrin from keratohyalin granules and subsequent interaction with keratin filaments are one of the final steps in the terminal differentiation of epidermal keratinocytes.

Keratinocyte differentiation and skin architecture are altered during 2-stage chemical carcinogenesis as well as after a single application of TPA (27, 28, 31, 33). Since filaggrin is potentially a marker for keratinocyte differentiation, it was of interest to observe filaggrin distribution in carcinogen-treated skin. This paper reports the isolation and characterization of filaggrin from mouse epidermis, the presence of an epidermal protein kinase which can phosphorylate filaggrin in vitro, and the altered staining pattern of filagrin in mouse papillomas and carcinomas during a standard 2-stage chemical carcinogenesis protocol.

MATERIALS AND METHODS

Animals and Chemicals. The mice used in these experiments were 6- to 8-week-old SENCAR mice bred at Oak Ridge National Laboratory. For the tumor studies, female SENCAR mice were initiated with a topical application of 10 mmol dimethylbenz[a]anthracene (Sigma Chemical Co., St. Louis, MO) and promoted twice weekly with 2 μg TPA in acetone (Dr. Peter Borchert, Minneapolis, MN) for 15 to 40 weeks.

For the biochemical studies, high-specific-activity γ[32P]ATP (3000 Ci/mmol) was purchased from Amersham/Seearle Corp. (Arlington Heights, IL); polyacrylamide gel reagents were from Bio-Rad Laboratories (Richmond, CA); CNBr was from Aldrich Chemical Co. (Milwaukee, WI); and bis-Tris was from Sigma. For the immunological studies, adjuvant was purchased from Difco Laboratories, Inc. (Detroit, MI), and preimmune goat serum and fluorescein isothiocyanate-coupled goat anti-rabbit serum was purchased from Amersham.

Polyacrylamide Gel Electrophoresis and Protein Purification. Analytical polyacrylamide gel electrophoresis in the presence of SDS-gels was done according to the method of Laemmli (21) with a final acrylamide concentration of 10%. Filaggrin was purified by the method of Steinert et al. (36) with modifications. The urea extract (5 μl urea-0.1 μl Tris (pH 7.5)-1 μm phenylmethylsulfonyl fluoride) from newborn mouse epidermis was mixed batchwise with DEAE-cellulose to absorb keratin proteins. That supernatant was either dialyzed and concentrated in acid-urea sample buffer (0.9 n acetic acid-9 μl urea) or dialyzed, lyophilized, and redissolved in Laemmli sample buffer (21). Preparative gels were run with thicker spacers (9 mm versus 1.5 mm) and loaded with up to 2 ml of a 10- to 20-mg/ml protein solution in Laemmli sample buffer (21). Gels were stained for 15 min in 0.2% Coomassie blue in H2O. After destaining overnight in water, the appropriate band was cut out, and the protein was recovered by electrophoresis through a 1-mm gel plug of 5% acrylamide into a dialysis bag. After dialysis, SDS and Coomassie blue were removed by ion-pair extraction (18). A similar procedure was developed

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utilizing 10% polyacrylamide acid-urea gels in place of SDS-gels (25, 30). The use of acid-urea gels eliminated the need for the ion-pair extraction. The final step of purification was by gel exclusion chromatography on a Biogel P6 column in 5% formic acid.

**Filaggrin Modification.** In *in vitro* phosphorylation, filaggrin was dissolved in PBS at 1 mg/ml. The protein kinase was partially purified from a 1 M phosphate extraction of mouse papillomas by column chromatography with phosphocellulose and DEAE-cellulose (24, 26). This kinase is cysic nucleotide independent, can use GTP as the phosphate donor, and is sensitive to heparin, similar to the kinase activity designated Casein Kinase II or G (6, 16, 17, 24). In a reaction volume of 100 µl, the final concentration of filaggrin was 0.1 mg/ml with 10 mM MgCl₂, 15 mM bis-Tris (pH 7.4), 70 mM NaCl, 2 mM dithiothreitol, 10 µM ATP, and 2 µCi γ-[32P]ATP. The reaction was started by the addition of 30 µl of kinase (specific activity, 2000 cpm/mg casein/min). Under these conditions, the reaction was linear for 1 hr at 37° (26, 29). For higher specific activity of labeling for gel analysis, the cold ATP was omitted, and reaction was terminated with SDS sample buffer after 20 min at 37°.

Filaggrin is resistant to CNBr cleavage, because it lacks methionine (23). CNBr cleavage was performed on skin extracts in 70% formic acid with a 500-fold excess of reagent for 2 hr at room temperature in the dark under N₂. Reactions were terminated by dilution with 4 volumes of H₂O and then lyophilized. Controls were treated identically but without CNBr.

**Immunological Techniques.** Filaggrin (0.5 mg/0.5 ml PBS) was emulsified with 2.5 ml Freund's complete adjuvant and injected subcutaneously into 3 sites on the back of a New Zealand White rabbit. Seven days after the third weekly immunization, the serum of the rabbit was obtained, and the IgG fraction was precipitated by adding ammonium sulfate to a final concentration of 45%. The precipitate was dissolved and dialyzed against 10 mM K₂HPO₄ (pH 8.0) and loaded onto a DEAE-cellulose column equilibrated in the same buffer (32). The eluted peak was concentrated to the original protein concentration by ultrafiltration. The preimmune serum was treated similarly. All studies reported here are with the DEAE-cellulose-purified IgG fraction.

Ouchterlony double-diffusion analysis was performed as described previously using 1.0% agar in PBS (32). Immunofluorescence studies were done essentially as described by Holbrook et al. (19). Both frozen and paraffin sections were used giving analogous results. Controls included samples incubated with preimmune serum and no serum. The “Western” immunoblot technique involving the electrophoretic transfer of protein from SDS-polyacrylamide gels to nitrocellulose was performed essentially as described by Burnette (5).

**RESULTS**

**Properties of Purified Filaggrin.** Filaggrin was purified from newborn mouse epidermis by a modification of the procedure of Steinert et al. (36). The purified protein migrated as a single band with an apparent molecular weight of 28,000 on SDS-polyacrylamide gels (Chart 1). The amino acid composition is very similar to other epidermal proteins isolated from rat, guinea pig, and 2 other strains of mice (Table I). The composition of filaggrin is very striking with the relatively large amounts of arginine and histidine and negligible lysine. In addition, filaggrin lacks methionine, tryptophan, and cysteine with very low levels of threonine, isoleucine, tyrosine, and phenylalanine. Serine, glycine, glutamic acid, and glutamine represent more than 50% of the protein.

Chart 1 demonstrates other properties of this filaggrin preparation. The SDS-gel in Chart 1A shows that filaggrin is stable during the 20-min incubation at 37° with a highly purified epidermal protein kinase (Lanes 2 and 3). The protein kinase is cyclic nucleotide independent and very similar to the kinase "CKII" or "G" described by others (6, 16, 17, 24). As reported for rat filaggrin, mouse filaggrin also acts as a phosphate acceptor in the *in vitro* kinase reaction (26). Chart 1B shows the cpm profile of [32P]P from the gel in Chart 1A, Lanes 3 and 4. Major peaks of [32P]P migrate in the same position as filaggrin as well as the position for the M, 24,000 kinase subunit (6, 17).

Chart 1A, Lane 8, also demonstrates the resistance of filaggrin to CNBr treatment, which is expected due to the absence of methionine (Table I). Epidermal 8 M urea-0.1 M Tris (pH 7.4) extracts were treated in parallel with and without CNBr to cleave methionine-containing proteins (Chart 1A, Lanes 6 and 7). Note that polypeptides of higher molecular weight were also resistant to CNBr treatment (Chart 1A, Lane 7).

**Specificity of Antifilaggrin Antibodies.** In order to examine the localization of the protein filaggrin in the mouse epidermis,
antibodies against filaggrin were raised in a rabbit. Antifilaggrin, but not control serum, gave a strong precipitation reaction with filaggrin in an Ouchterlony double-diffusion assay (Fig. 1). Preabsorption of the immune sera with filaggrin eliminated the precipitation reaction (data not shown). Preabsorbed antibody also resulted in decreased immunofluorescence in papillomas (see below; Fig. 5D).

To further categorize the specificity of the antifilaggrin antibody preparation, Western blot analysis was performed on 8 M urea extracts from adult mouse epidermis and newborn mouse epidermis treated with CNBr. The 8 M urea-0.1 M Tris (pH 7.5) adult mouse extract was separated on a 10% polyacrylamide-SDS gel, and the Coomassie blue-stained gel is shown in Fig. 2a. A similar gel was used to electrophoretically transfer protein to a nitrocellulose filter which was subsequently incubated with antifilaggrin antibody and developed with 125I-labeled protein A. The resulting autoradiogram is presented in Fig. 2b. In the adult mouse skin extract (Lane 2), the M, 28,000 form of filaggrin is observed as well as immunocross-reactive species that are approximately 2 and 3 times the molecular weight of filaggrin. In addition, a higher-molecular-weight form is observed that just enters the 10% polyacrylamide gel. Fig. 2c compares the immunoblot pattern of the 8 M urea-0.1 M Tris (pH 7.5) extract before and after CNBr treatment. The CNBr-treated sample gave essentially the same pattern of higher-molecular-weight cross-reacting species.

**Survey of Mouse Epidermis by Indirect Immunofluorescence.** The rabbit antifilaggrin antibody was used in an indirect immunofluorescent assay with fluorescein isothiocyanate-coupled goat anti-rabbit IgG so that frozen sections of mouse epidermis could be examined for cellular localization of filaggrin. In Fig. 3A, the frozen section of skin from a 6- to 8-week-old SENCAR mouse was examined using this technique. The granular layer was strongly positive with very weak reaction observed in the basal and spinous layers as well as the stratum corneum of the epidermis. If preimmune serum was substituted for the antifilaggrin serum, or if the antifilaggrin serum was omitted, then no immunofluorescence was observed (Fig. 3C). When newborn SENCAR mouse skin was examined with the antibody preparation (Fig. 3B), significant immunofluorescence was observed in the stratum corneum, similar to patterns reported by others (10, 14, 19).

Filaggrin distribution was determined by the immunofluorescent technique in 6- to 8-week-old SENCAR mice 48 hr after topical TPA treatment. After a single TPA treatment (Fig. 4A), spinous and basal cell layer fluorescence is markedly enhanced in addition to granular layer fluorescence. In Fig. 4A, certain basal and spinous cells appear strongly fluorescent and may represent a wave of cells stimulated by TPA differentiate. This pattern is not as pronounced, however, in the epidermis of mice treated 3 times with TPA over a 2-week period (Fig. 4B). The pattern after...
multiple treatments of TPA shows less basal and spinous cell fluorescence. The skin is considerably thicker with multiple layers of granular cells visualized with the immunofluorescent technique. With multiple TPA treatments, the hyperplastic epidermis and thickened granular layers are similar to the epidermis of newborn animals, although with reduced immunofluorescence in the stratum corneum.

Papillomas are the benign form of skin tumors that are observed after 10 to 20 weeks of the dimethylbenz[a]anthracene initiation-TPA promotion protocol in the SENCAR mouse. The number of papillomas correlates well with the probability of invasive carcinomas developing after 30 or more weeks of treatments (35). Papillomas and carcinomas produced in SENCAR mice by the initiation-promotion protocol were also examined with the indirect immunofluorescent technique, and the results are presented in Fig. 5. In general, most papillomas examined showed increased antifilaggrin fluorescence in all layers. Smaller papillomas had primarily enhanced granular cell immunofluorescence (Fig. 5B; Table 2). Larger, exuberant papillomas showed a very pleomorphic distribution of filaggrin with positive areas alternating with negative ones. Occasionally, single highly fluorescent basal cells were observed (Fig. 5C). Preabsorption of the antisera with purified filaggrin markedly reduced the amount of observable fluorescence in these papilloma samples (Fig. 5D).

Invasive carcinomas produced by the same initiation-promotion protocol were negative or only slightly immunofluorescent positive. In Fig. 6, one can compare the fluorescence of an invasive squamous cell carcinoma with the overlying epidemis with its positive granular layer. The fluorescence of the carcinoma is relatively weak, similar to the basal and spinous layer of cells.

DISCUSSION

Evidence that the protein purified from mouse epidermis is indeed filaggrin is based upon: (a) solubility properties; (b) amino acid composition; (c) resistance to CNBr cleavage; (d) immunofluorescent localization; and (e) in vitro substrate in a phospho-rylation reaction. Proteins that comigrate with mouse filaggrin on SDS-gels were present in 8 M urea extracts with and without β-mercaptoethanol as well as in 1 M phosphate, extraction procedures which others have shown to solubilize filaggrin (1, 3, 8, 11, 34, 36). The amino acid composition is perhaps the most striking identifying characteristic for filaggrin as well as a criterion for purity (i.e., no methionine or cysteine). Since only 6 amino acids (serine, glutamic acid, glutamine, glycine, alanine, and arginine) account for more than 75% of the protein, this protein behaves differently in solution than do globular proteins. Solubility problems, aggregation, and ambiguities in molecular weight determination are all problems in working with this protein. For instance, quantitation using classical immunochromical techniques has been hampered by the requirement of high phosphate or urea concentrations necessary for maintaining solubility of epidermal extracts.

The specificity of the rabbit antiserum against the SDS-polyacrylamide gel electrophoresis-purified M, 28,000 form of filaggrin was determined by several different experimental approaches. (a) Preimmune sera gave negative results (Figs. 1 and 3). (b) Preabsorption with purified filaggrin virtually eliminated immunofluorescence in papillomas (Fig. 5). (c) The multiple cross-reactive species detected by the immunoblot technique were CNBr resistant (Fig. 2c), and although the cross-reactive species in the M, 50,000 to 65,000 range of the gel varied from preparation to preparation, the amount of antigen present was not dependent on the concentration of keratin. However, it is not possible to state that the antibody preparations are monospecific for filaggrin; contaminating antibodies towards other proteins such as keratins are possible, albeit in low titer.

Lonsdale-Eccles et al. (23) used CNBr and a 2-dimensional polyacrylamide gel technique to examine skin from different species for the presence of filaggrin and found that the molecular weights ranged from about 27,000 to 60,000, depending on the species of origin. Since the amino acid compositions from various species are similar and relatively simple with possibly a repeating sequence, heterogeneity in the number of repeats may yield different molecular weights but functionally identical proteins. Alternatively, if filaggrin exists in a high-molecular-weight precursor form (4, 15, 34, 37), then these differences may result from slightly altered proteolytic cleavage patterns. Protein with molecular weights 2 or 3 times that of filaggrin have been observed to be resistant to CNBr treatment (Chart 1A, Lane 7; Ref. 23). In addition, similar protein bands of multiple molecular weight are immunologically cross-reactive and essentially unchanged after CNBr treatment (Ref. 19; Fig. 2). It is unclear whether these bands represent a nonspecific aggregation or discrete steps in the processing of filaggrin from or to its high-molecular-weight forms (1, 4, 15, 19, 22, 32, 35). Ugel (37) has also described oligomers of ribonucleoproteins from keratohyalin, and often these oligomeric proteins form doublets on SDS-polyacrylamide gels.

During terminal differentiation in the skin, synthesis of filaggrin is greatly enhanced in the granular cells. What triggers this process is unknown. Basal cells break their attachment through hemidesmosomes to the basement membrane and form more desmosomes in the stratum spinosum. During this process, significant changes in the intermediate filaments associated with desmosomes would be expected to occur. The intermediate filament composition is changing as different keratin proteins (i.e., the appearance of a high-molecular-weight keratin band of around 67,000) are synthesized during terminal differentiation (12, 13). Stimulation of filaggrin synthesis is probably linked in some way to these changes; TPA may accelerate these changes in certain basal cells, resulting in the fluorescence data presented in Fig. 4A.

An increase in filaggrin immunofluorescence was correlated with hyperplasia induced by TPA in the initiation-promotion protocol for production of skin tumors (Fig. 4). This is in good agreement with the results of Balmain (2, 3), which show that TPA stimulates the synthesis of a M, 27,000 protein in mouse epidermis with essentially the same amino acid composition as filaggrin (Table 1). Balmain (2, 3) observed the greatest stimulation at 48 to 72 hr after treatment, which is similar to our results.

In large, exuberant papillomas, filaggrin distribution has dramatically changed with areas of high and low immunofluorescence as well as a generally disrupted pattern. In some ways, this disorganized pattern of antifilaggrin immunofluorescence is similar to that reported by Holbrook et al. (19) in skin from the repeated epilation (er/er) mutant mouse. Papillomas with an altered filaggrin distribution, as determined by immunofluorescent techniques, are γ-glutamyltransferase positive (Table 2) and have decreased levels of high-molecular-weight keratin (20, 27,
In carcinomas, which are all \( \gamma \)-glutamyltransferase positive and lack high-molecular-weight keratin, there is little or no filaggrin detectable. Therefore, during the carcinogenic process, 2 structural proteins, the synthesis of which is greatly enhanced during terminal differentiation, are markedly reduced. Preliminary studies with human skin tumors corroborate the mouse studies. \(^5\) Human invasive squamous carcinomas of the skin showed little immunofluorescence, whereas most keratoacanthomas contained filaggrin in the cornified cells.

In conclusion, filaggrin appears to be a good indicator of maturation and differentiation in experimental and human epidermal tumors, and its presence correlates well with the absence of aggressive or invasive growth. These findings, as well as the molecular biology of filaggrin synthesis and processing and its role in normal cellular morphology, merit considerable investigation in the future.

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Fig. 4. Indirect immunofluorescence of TPA-treated mouse epidermis. Samples were treated as described in Fig. 2. d, dermis. A, 8-week-old mouse epidermis 48 hr after topical application of 2 μg TPA in acetone. × 350. B, 48 hr after third application of TPA, 2 μg every third day. × 350.

Fig. 6. Indirect immunofluorescence of a squamous cell carcinoma (CA). Treated as described in Fig. 4. Arrows, positive reaction in granular layer of overlying epidermis (EP). × 150.
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Fig. 5. Indirect immunofluorescence detected on paraffin sections of papillomas with rabbit anti-filaggrin antibody. Samples treated as in Fig. 3. CT, connective tissue; HL, horny layer. A, contiguous section of a small papilloma. Note central connective tissue axis (CT) and abundant horny material (HL). H & E, x 120. B, section of small papilloma. x 150. C, section of large papilloma. →, positive reaction in some basal cells. x 150. D, cross-section of a papilloma stained with antifilaggrin serum preabsorbed with purified filaggrin. Immunofluorescence is markedly reduced. x 180.
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