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The presumption of proteolytic enzymes related to the formation of intermediates during the terminal differentiation

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The [^{14}C]Gly-labelled keratin polypeptides extracted with 1% SDS and 10 mM DTT were made to undergo changes with an enzyme fraction (ammonium sulfate, 50–75% saturated fraction) prepared from a human epidermis in the presence of 1% Triton X-100. In particular, 69–67 kDa peptides were considerably decreased with the above enzyme fraction in the time course experiments, and the components strongly bound to the cell membrane had little effect on the above reaction. In addition, in the case of the [^{14}C]Gly-labelled keratin filament assembly, 69 and 62 kDa peptides were decreased and 55, 52 and 50 kDa peptides were increased with the same enzyme fraction in the time course experiments. From these results, we estimated that the proteolytic enzyme(s) may exist in the human epidermis, and may be processed to keratin intermediates from prekeratin during the initial stage of terminal differentiation in the human epidermis.

Keratin; Processing enzyme; Intermediate; Terminal differentiation

1. INTRODUCTION

Keratin filaments represent about 20 different subunits found in keratinocyte and other epithelial cells [1–4]. In addition, the filaments are divided into two classes of subunits, types I and II, ranging in molecular mass from about 40 to 70 kDa and in isoelectric value from pH 5 to 8 [3,5].

Recently, we indicated that keratin peptides (the filament protein of the stratum corneum) were processed by way of two kinds of keratin intermediates from prekeratin (the tonofilament protein of the living epidermis) with some proteolytic enzyme(s) during the initial stage of epidermal differentiation in the human epidermis [6,7] and an epidermal disease [8].

In this paper, as there is little information on the particular proteases related to the keratin peptides in the human epidermis [9], we wanted to demonstrate whether [^{14}C]keratin polypeptides and/or the [^{14}C]keratin filament assembly could react with the proteolytic enzyme(s) fraction partially fractionated from the human epidermis.

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Abbreviations: SDS, sodium dodecyl sulfate; DTT, dithiothreitol; SDS-buffer, 62.5 mM Tris-HCl (pH 6.8)/2% SDS/10% glycerol/5% β-mercaptoethanol

2. MATERIALS AND METHODS

[U-^{14}C]Glycine (110 mCi/mmol) and EN^{3}HANCE were purchased from New England Nuclear, Boston, MA. The extraction method of [^{14}C]Gly-labelled keratin was carried out as described [7], and the [^{14}C]keratin filament assembly was prepared using the method described in [10].

2.1. *Preparation of the enzyme fraction*

A normal human epidermis (about 4.5 g wet wt) was homogenized with 40 ml of 0.1 M Tris-HCl (pH 7.6)/0.1 M NaCl/1% Triton X-100 using a glass homogenizer. The homogenate was then filtered through three layers of gauze and filtrate thus obtained was centrifuged at 700 × g for 10 min. The supernatant subsequently obtained was further centrifuged at 14000 × g for 30 min. In addition, the supernatant was centrifuged at 28000 × g for 1 h to yield the enzyme solutions. To fractionate the enzyme solutions obtained into the three fractions, solid ammonium sulfate was added to the solutions. The resulting precipitations were designated fractions I, II and III, respectively (I, 0–25% sat.; II, 25–50% sat.; III, 50–75% sat.).

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On the other hand, the residue obtained after centrifugation (700 × *g*, 10 min) was suspended with 0.1 M Tris-HCl (pH 7.6)/1% SDS for 3 h, and the suspended solution was then centrifuged at 40000 × *g* for 30 min. The supernatant thus obtained was dialyzed against water and lyophilized (this component was designated letter P).

2.2. *Enzyme reaction and SDS-PAGE*

The [^{14}C]keratin peptides (about 25–50 μg/10 μl) extracted as in [7] were incubated with the above enzyme fractions (I–III, about 1–2 μg/10 μl, each fraction) at pH 8.5 for 0–3 h at 37°C. The protein concentration was determined with the method used in [10]. After incubation, the reaction mixture was boiled at 100°C for 5 min to stop the enzyme reaction and was then centrifuged at 15000 rpm for 10 min. The supernatant thus obtained was applied to SDS-PAGE (containing 10% acrylamide) as in [11], followed by fluorography as in [12], using EN^{3}HANCE. Each band of the SDS-PAGE gel which corresponded to one in the fluorographic X-ray film was cut out and solubilized with 30% H_2O_2 at 60°C for 6 h, and counted by a liquid scintillation counter. In some experiments, the component P in the presence of 0.1% Triton X-100 was further added to the above reaction mixture.

3. RESULTS AND DISCUSSION

As for the post-translational modification during human epidermal differentiation, no information is available, with the exception that keratin peptides may be modified in the granular cell layers as in [13] and a precursor-product relation may exist between the spinous layer and the stratum corneum layer keratins [9,13].

To address this fundamental question, the labelled keratin peptides were allowed to react with the partially fractionated enzyme fractions I–III prepared from the human epidermis. From the results of fluorography of SDS-PAGE after the reaction with the above enzyme fractions, fraction III showed images of the 69–67 kDa keratin peptides having been considerably decreased after incubation for 3 h. However, fraction I did not affect any changes on the keratin peptides, while fraction II showed drastic and random cleavages for keratin peptides (not shown). The modified (or cleaved) reaction of 69–67 kDa keratin peptides progressed further in the presence of component P as shown in fig.1. As a comparison with the control fraction (to deactivate enzyme fraction III at 100°C for 10 min), fraction III effected some changes on the keratin peptides. In particular, 69–67 kDa peptides were considerably decreased. Furthermore, 58–52 kDa keratin peptides also

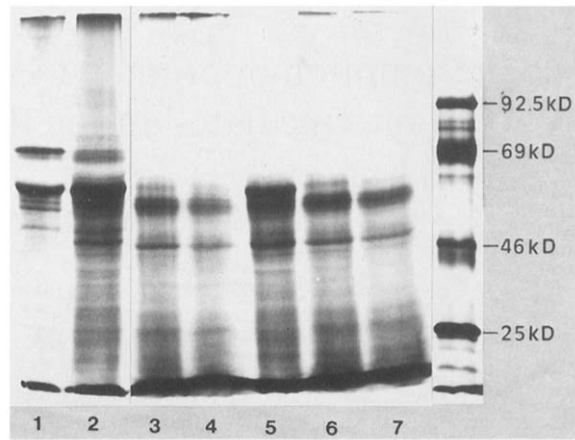

Fig.1. Fluorographs of SDS-PAGE after the incubation of [^{14}C]keratin peptides and enzyme fraction III. Lanes: 1, fraction III (1–2 μg/10 μl) was boiled at 100°C for 10 min, deactivating it, and then was added to [^{14}C]keratin peptides (about 25–50 μg/10 μl); 2, fraction III + [^{14}C]keratin peptides; 3, 2 +component P/10 mM Tris-HCl (pH 7.6)/0.1% Triton X-100 (protein conc., about 100 μg/10 μl); 4, 2 + 3 vols of component P; 5, 2 vols of fraction III + [^{14}C]keratin peptides; 6, 5 + 3 vols of component P. Fraction nos 1–7 were incubated at 37°C for 3 h and then applied to SDS-PAGE.

showed little change in the presence of component P as shown in fig.1 (lanes 3,4,6 and 7). In accordance with the reaction time, 48–46 kDa keratin peptides appeared as shown in fig.1 (lanes 2,3,5 and 6).

To make sure that the results described above are due to the presence of proteolytic enzymes in the human epidermis, the time course with fraction III was demonstrated and presented as shown in fig.2. As a result of the precipitation during incubation, the reaction mixtures were centrifuged at 15000 rpm for 10 min. From this profile of the supernatant within the reaction mixture (fig.2A), 69–67 kDa keratin peptides disappeared perfectly and keratin peptides of low molecular masses (49–46 kDa) were increased in accordance with the incubation time. On the other hand, the precipitation was further extracted with 4 M urea/SDS buffer/10 mM DTT at 100°C for 3 min. The extracted fraction was applied to SDS-PAGE and fluorography was performed. As shown in fig.2B, 69–67 kDa keratin peptides were presented in this fraction, although their molecular mass appeared to have slightly decreased. However, 49–46 kDa

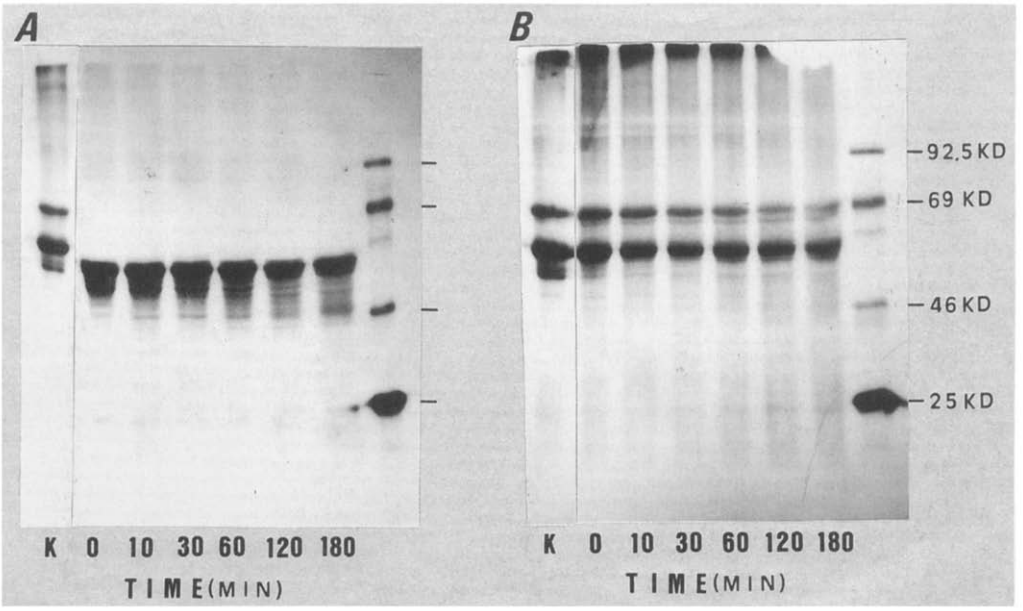

Fig.2. Fluorographs of SDS-PAGE after incubation during the time course of ^{14}C-labelled keratin peptides and enzyme fraction III. (A) The applied samples were supernatant of the reaction mixture during various lengths of time. (B) After incubation for 0–3 h, the resulting pellet was boiled with 4 M urea/SDS buffer/10 mM DTT at 100°C for 3 min. The extracted supernatant was applied to SDS-PAGE. K, keratin peptides only.

keratin peptides represented in the above supernatant fraction were not contained. These results may be due to changes in the solubility of keratin peptides against the solution employed. In other words, the terminal residues (N- and/or C-) of keratin peptides may be modified with the proteolytic enzyme(s) in fraction III, and the slightly alternated keratin peptides were then separated into the two fractions in which 4 M urea and 10 mM DTT were contained or not contained. In any event, we estimate that the keratin peptides may effect some changes with the proteolytic enzymes in fraction III during the initial stage of the processing of keratin intermediates.

In order to further elucidate the modified sites of keratin peptides with fraction III, the [^{14}C]keratin filament assembly was allowed to react with fraction III (keratin assembly/fraction III = about 100:1, w/w, from data as in [10]) for 0–4 h at 37°C. The [^{14}C]keratin filament assembly obtained using the method as in [14] was examined by electron microscopy. The picture of filament assembly was similar to that in [1] and in [15] (not shown). After the incubation of [^{14}C]keratin filament assembly and fraction III in the time course experiments, fluorography was performed as in [12]. Each band in the SDS-PAGE gel which corresponded to one in the fluorographic X-ray film was cut out and counted with a liquid scintillation counter as shown in fig.3. From 0 to 2 h of incubation, 62 and 69 kDa peptides tended to decrease, and 55, 52 and 50 kDa peptides were gradually increased. All keratin peptides showed little change after 2 h.

In keratin filament assembly and architecture, the N- and C-nonhelical termini may be predominantly located on the surface of protofilament and may be variable in length [16]. From the above facts and the data described here, one can conclude that some of the proteolytic enzyme(s), of which fraction III is comprised, may attack the terminal region(s) of keratin peptides. The above proteolytic enzyme(s) may therefore play a role in processing related to the post-translational modification or terminal differentiation in the human epidermis.

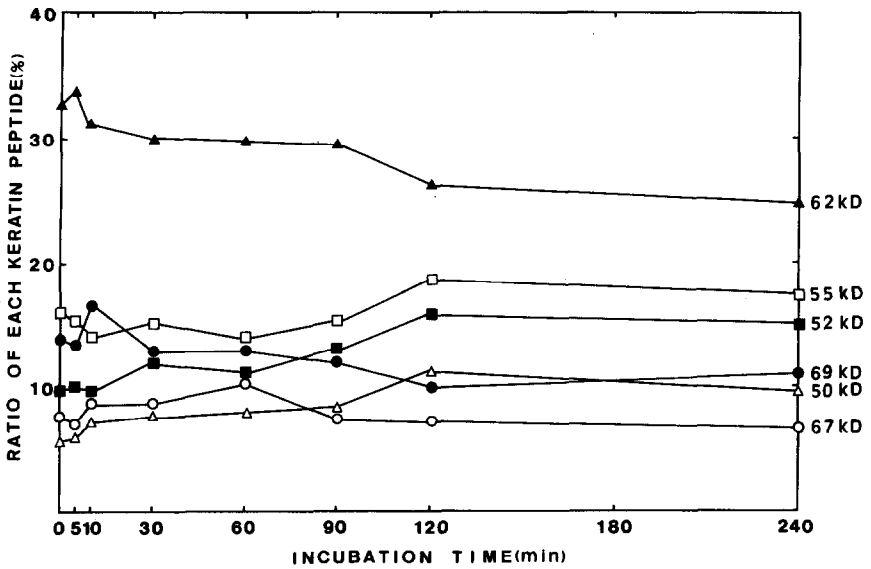

Fig.3. Radioactive changes during the time course of [^{14}C]keratin assembly and enzyme fraction III. Each band in the SDS-PAGE gel that corresponded to one in the fluorographic X-ray film was cut out and counted by a liquid scintillation counter. The total counts of each lane is 100%. Ratios of count in each are shown in this figure.

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