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Stepwise Modifications of Keratin Polypeptides during Keratinization in Palmar–Plantar Epidermis

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Precursor–product relationships among keratin polypeptides (cytokeratins) were studied in living and cornified layers of palmar–plantar epidermis. Serial 10 μm horizontal freeze-cut sections of punch biopsies were analysed by means of light microscopy and one-dimensional electrophoresis in a system that permitted identification of all major keratin polypeptides with apparent molecular weights over 60 kDa. Peptides that appeared to be related were compared by means of peptide mapping after partial proteolysis.

The results suggest that both the basic-neutral (type II) cytokeratin no. 1 and the acidic (type I) cytokeratin no. 9 undergo two distinct modification steps with subsequent decreases in apparent molecular weight during keratinization. For both polypeptides the first modification appeared to take place and run to completion in close relation to the transition between the uppermost living epidermal layers and the lowest cornified layers. The second conversions of cytokeratins nos. 1 and 9 both appeared to take place within the stratum corneum but differed in two respects: 1) they appeared to start at different tissue sites; 2) whereas the second modification step appeared to comprise all cytokeratin 1 molecules, only a fraction of the cytokeratin 9 molecules passed through this step. These variations suggest that the different modification steps may be produced by different mechanisms that are regulated separately. It is concluded that the processing of cytokeratins during keratinization may be more complex than has previously been realized.

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According to current models of keratin intermediate filaments, the end regions of the cytokeratins (keratin polypeptides) are situated on the surface of the filaments. In this way the tissue-specific cytokeratin composition of a given epithelium may give the intermediate filaments certain surface properties that are important for the function of this particular tissue (1). This may be of importance in epidermis where the keratin filaments come to be arranged into a typical 'keratin pattern' (2, 3) during the transition from living to cornified layers. In this process the interaction between filaments and specific proteins, filaggrins (4), may be important. It is also possible, however, that the aggregation of the filaments is induced or facilitated by changes in their surface properties, viz. modifications of the end regions of cytokeratins. Such modifications could be induced for example by proteolytic cleavages (5). There are a number of reports presenting evidence that many of the cytokeratins found in the stratum corneum have been proteolytically modified during the keratinization process (6–11).

The first evidence of proteolytic modifications of cytokeratins during terminal differentiation of palmar–plantar epidermis was presented by Fuchs & Green (6). They analysed the cytokeratin composition of serial horizontal slices of plantar skin by means of one-dimensional polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS–PAGE) and found an apparent decrease in molecular weight of the largest cytokeratin in the stratum corneum as compared with layers of living epidermis.

The present work used the experimental set-up of Fuchs & Green (6) combined with a modified one-dimensional SDS–PAGE system that considerably improved the resolution of the high-molecular weight cytokeratins of suprabasal palmar–plantar epidermis. It was possible to follow the fate of the two major cytokeratins of palmar–plantar epidermis (the basic-neutral type II cytokeratin no. 1, K1, and the acidic type I cytokeratin no. 9, K9) (11–13) during the transition from living to cornified layers and in the different layers of the stratum corneum. The results show that both polypeptides are subject to at least two distinct modification steps during this development and that the different steps take place at different tissue sites. Thus the modification of cytokeratins during keratinization may be more complex than has previously been realized.

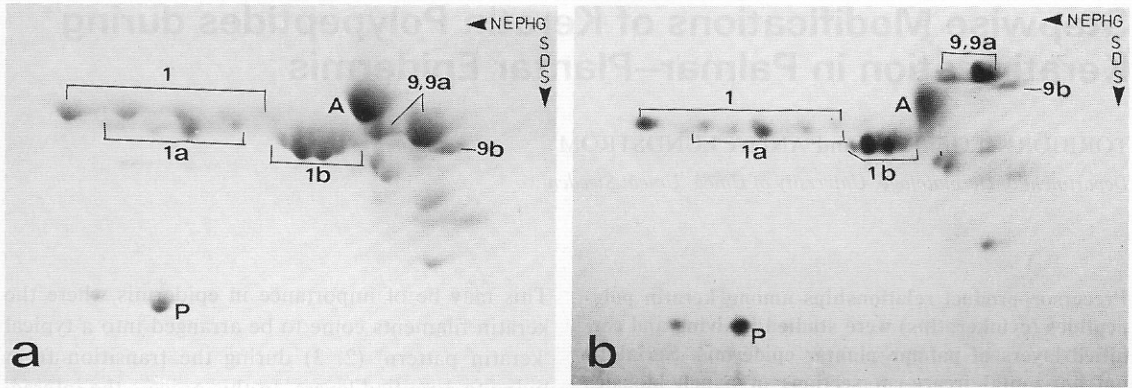

Fig. 1. Two-dimensional electrophoresis of keratin polypeptides from whole palmar epidermis (living + cornified layers). The numbering of keratin polypeptides ("*1*" and "*9*") was adopted from (12). The letters '*a*' and '*b*' denote suggested derivatives of keratin polypeptides no. 1 and 9 that have passed through the first and second modification steps respectively (see text). K1a and K1b are probably identical with "*1**" and '*X*' respectively in ref. no. 11. '*A*' and '*P*' standard proteins; *A* = bovine serum albumin (Mw 66 kDa, isoelectric point of major variant 6.35), *P*=phosphoglycerokinase (Mw 43 kDa, isoelectric point of major variant 7.4). In (*a*), SDS–PAGE was run in the Laemmli system; in (*b*) in our modified system (see text). 10% gels in both systems. Note the different mobilities of keratin peptide no. 9 and its suggested derivatives in the two systems.

MATERIALS AND METHODS

Four-mm punch biopsies were taken from the proximal-ulnar aspects of the palms of volunteers with normal skin or from the mid-lateral part of the soles of autopsy cases. For the preparation of extracts of whole epidermis, biopsies were freed from most of the dermis under a dissection microscope, minced, and extracted in lysis buffer (14) for 15 h at 37°C. For horizontal slicing, biopsies were frozen on dry ice, care being taken to obtain a horizontal upper surface. Serial 10-µm sections parallel to the skin surface were prepared in a cryostat with the biopsies mounted with the skin surface towards the knife. Sections nos. 1, 3, etc. were extracted in 50 µl of Laemmli's sample buffer (15) on a boiling-water bath for 2 min. Sections 2, 4, etc. were mounted on glass slides and stained with haematoxylin and eosin. Serial sectioning and subsequent electrophoretic and morphological analyses were performed on four biopsies from the palms of 3 volunteers and two biopsies from the soles of autopsy cases.

Electrophoresis

Non-equilibrium, pH gradient electrophoresis (NEPHGE) in 180×1.7 mm glass tubes was performed as described by O'Farrel et al. (16) except that the ampholytes used were Pharmalyte® 3–10 (Pharmacia Fine Chemicals, Uppsala, Sweden). SDS–PAGE was performed in slab gels (180×200×0.7 mm for the second dimension in two-dimensional electrophoresis, 160×180×1.5 mm for one-dimensional analysis of keratin peptides, and 73×102×0.75 mm minigels (Mini Protean II, BioRad, Richmond, Calif.) for one-dimensional peptide mapping). The *buffer-gel systems* for SDS–PAGE were either Laemmli's (15) system, or a modified system. The *modified system* was that described by Thomas & Kornberg (17) and used by Moll et al. (18) but with the same electrode buffer as in the Laemmli system (0.025 M Tris, 0.19 M glycine, pH 8.3, 0.1% SDS). The further composition of this system was as follows: acrylamide/bisacrylamide 30/0.15; stacking gel—total acrylamide concentration 3%, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS; separation gel—total acrylamide concentration 10%, 0.75 M Tris-HCl pH 8.8, 0.1% SDS. Gels were run for 15–18 h at 10 mA/gel (Laemmli system) or 20 mA/gel (modified system). Minigels were run at 200 V for 45–60 min. Protein bands were visualized with Coomassie Blue.

Peptide mapping

Individual keratin polypeptides, separated in the modified SDS–PAGE system, were electrophoretically eluted in electrode buffer in a Model 422 Electro Eluter (BioRad) for 15 h at 8 mA/tube. The purity of the eluted polypeptides was checked by re-electrophoresis. One-dimensional peptide mapping was carried out *ad modum* Cleveland et al. (19) with staphylococcal V8-protease (ICN, Lisl, Ill.).

RESULTS

The experiment presented in Fig. 1 was performed in order to demonstrate the properties of the modified system for SDS–PAGE as compared with the conventional Laemmli system. (The numbering of individual cytokeratins was based on a synthesis of data from Moll et al. (11, 12) and data presented in this work. Modified polypeptides were given the numbers of their suggested mother polypeptides with subscripts '*a*' and '*b*' for the polypeptides that had passed through the first and second modification steps respectively.) The aberrant mobility of K9 and its sug-

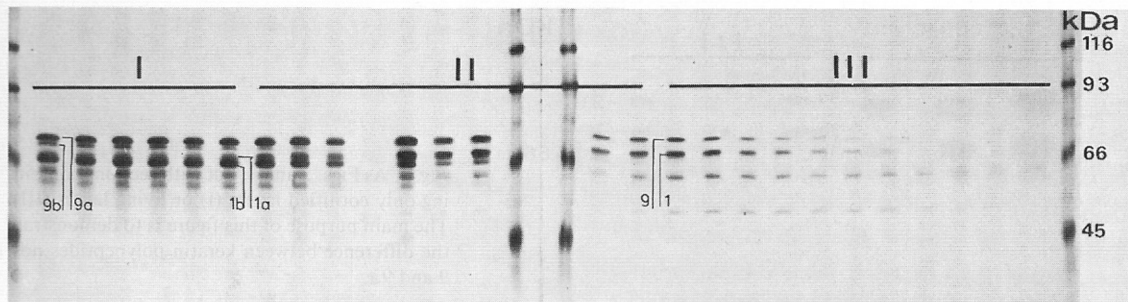

Fig. 2. SDS–PAGE analysis (modified system) of serial 10-µm freeze sections nos. 1, 3, etc. (from left to right) parallel to the skin surface of a 4-mm punch biopsy from the palm of an individual with normal skin. The samples of the two gels were surrounded by molecular weight markers. Sections 2, 4, etc. were examined by light microscopy. I=sections containing only cornified layers, II=sections containing decreasing amounts of cornified layers and increasing amounts of living layers. III=sections containing only living layers. The numbering of keratin polypeptides as in this figure was based on relative mobilities in the second dimension of two-dimensional electrophoresis (see Fig. 1 *b*). The empty lane under 'II' was due to a lost section. Similar results were obtained when four biopsies from the palms of 3 different individuals and two biopsies from the soles of autopsy cases were analysed.

gested derivatives in the modified system made it possible to identify polypeptides relevant for this study after a one-dimensional run. This is further demonstrated in Figs. 2–4.

Evidence of precursor–product relationships among cytokeratins based on analysis of serial sections

The principle for the type of experiment presented in Figs. 2–4 was adopted from the work of Fuchs & Green (6). Horizontal 10-µm cryostat sections were taken from punch biopsies of normal palmar epidermis. Every second section was mounted for light microscopical examination, whereas extracts of sections 1, 3, etc. were analysed by one-dimensional SDS–PAGE in the modified system. Due to the undulating transition zone between living and cornified epidermal layers, a series (II in Figs. 2 and 3) of sections was obtained that contained gradually decreasing amounts of stratum corneum and increasing amounts of living layers. Due to the anatomy of the basal membrane zone with the dermal papillae rising into the epidermis, sections from deeper layers contained gradually increasing amounts of basal layer and connective tissue. Fig. 2 shows gels with extracts of serial sections from the entire epidermis. In Fig. 3, extracts of serial sections from the transition between living and cornified layers were surrounded by extracts from two levels of stratum corneum (to the left) and one extract from a section with only living layers (to the right). In Fig. 4, extracts from superficial stratum corneum were compared with extracts from living layers in order to show the differences between unmodified and finally modified keratin polypeptides nos. 1 and 9.

When the gels were analysed from the right to the left, i.e. from the living layers towards the stratum corneum, the following conversions of K1 and K9

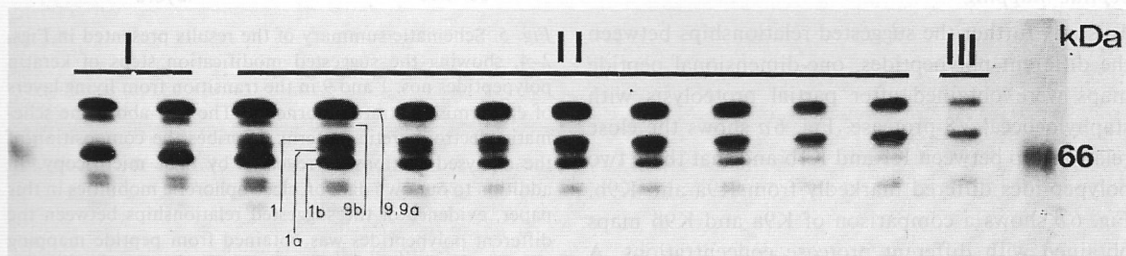

Fig. 3. As Fig. 2. Some sections containing either only cornified layers or only living layers were omitted in order to make it possible to demonstrate more clearly the events in the transition between living and cornified layers in the same gel.

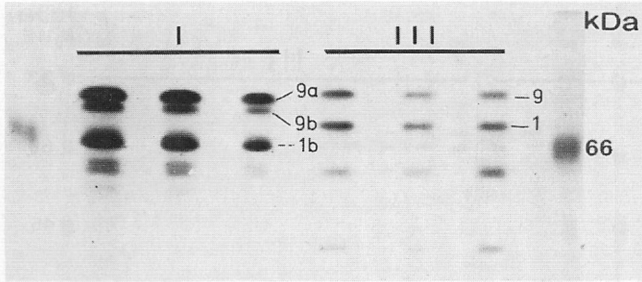

Fig. 4. As Figs. 2 and 3, but with sections containing only cornified layers (I) or living layers (III). The main purpose of this figure is to demonstrate the difference between keratin polypeptides nos. 9 and 9a.

(schematically summarized in Fig. 5) appeared to have taken place: K1->K1a->K1b; K9->K9a->K9b. (In this interpretation it was assumed that, since K1a-b and K9a-b were present only in sections containing stratum corneum, these polypeptides had not been synthesized *de novo* but must have been the result of post-translational modifications.) K1->K1a and K9->K9a appeared to have taken place at the same site and early on in the keratinization process, in close relation to the transition between the granular layer and the lowest layer of the stratum corneum. K1a->K1b and K9a->K9b appeared to have been initiated at different tissue sites and differed also in the extent to which they had been completed within the stratum corneum. Whereas the production of K1b appeared to start at the same site as the production of K1a and K9a, K9b could be first detected higher up in the stratum corneum. All K1a appeared to have been converted to K1b within the stratum corneum. In contrast, K9a could be found in all sections, suggesting that the second conversion of K9 comprised only a fraction of the molecules. Results very similar to those presented in Figs. 2–4 were obtained when the analyses were repeated on biopsies from the palms of various individuals or from the soles of autopsy cases.

Evidence for precursor–product relationships among cytokeratins based on peptide mapping

To study further the suggested relationships between the different polypeptides, one-dimensional peptide maps were obtained after partial proteolysis with staphylococcal V8-protease. Fig. 6*a* shows the close relationship between K1 and K1b and that these two polypeptides differed markedly from K9a and K9b. Fig. 6*b* shows a comparison of K9a and K9b maps obtained with different protease concentrations. A majority of the peptides that had been produced during the proteolytic degradation of the two polypeptides had identical electrophoretic mobilities, suggesting a close relationship.

DISCUSSION

The results presented in this paper provide a further insight into the complexity of the mechanisms by which the processing of keratin polypeptides in the final steps of epidermal differentiation occurs.

The stepwise modification of K1 and K9 during keratinization has not been described before. The modified electrophoresis system that allowed identification of individual high molecular weight keratin polypeptides after a one-dimensional run was of crucial importance to this work. With the frequently used Laemmli system (15), as in the work of Fuchs & Green (6), the only detectable event would have been an apparent disappearance from the stratum corneum

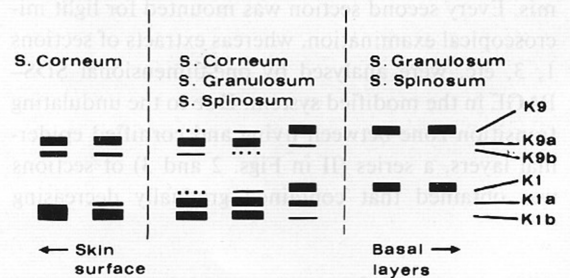

Fig. 5. Schematic summary of the results presented in Figs. 2–4, showing the suggested modification steps of keratin polypeptides nos. 1 and 9 in the transition from living layers of epidermis to stratum corneum. The text above the schematic electrophoretic patterns describes the composition of the analysed sections as revealed by light microscopy. In addition to our own data on electrophoretic mobilities in this paper, evidence of the suggested relationships between the different polypeptides was obtained from peptide mapping (ref. 11 and this paper) of K1, K1a and K1b and of K9a and K9b. The relation K9->K9a was based solely on results from one and two-dimensional electrophoresis (this paper).

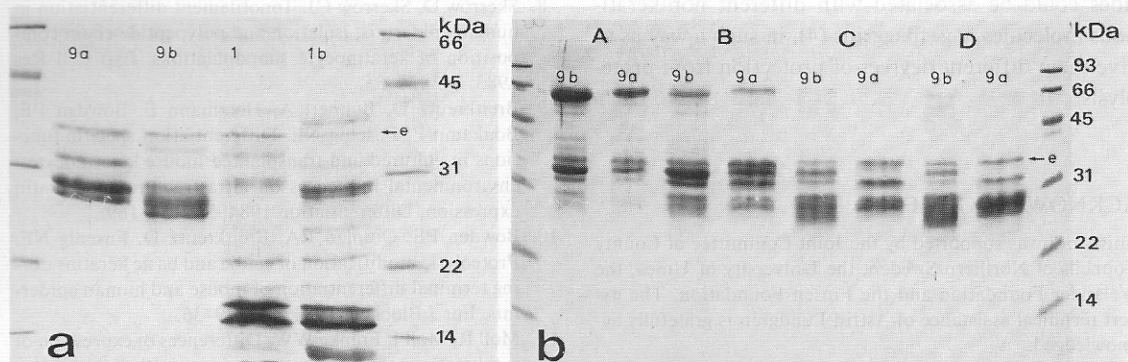

Fig. 6. One-dimensional peptide mapping with staphylococcal V8-protease of keratin polypeptides from palmar epidermis. Purified polypeptides (approximately 1 mg/ml) in electrode buffer (0.025 M Tris, 0.19 M glycine, 0.1% SDS, pH 8.3) were incubated for 30 min at room temperature with protease and then prepared for SDS–PAGE (15% gels, Laemmli system). In (*a*), keratin polypeptides 1 and 1b were compared with keratin polypeptides 9a and 9b, protease concentration 100 μg/ml. In (*b*), keratin polypeptides 9a and 9b were compared at different protease concentrations: $A=3$ μg/ml, $B=10$ μg/ml, $C=30$ μg/ml, $D=100$ μg/ml, '*e*' = V8-protease. The samples were surrounded by molecular weight markers.

of K1, since there is considerable overlapping between modified K1 and the different K9-peptides in this system. Of course, two-dimensional electrophoresis gives superior resolution of cytokeratins, but this technique would have posed serious practical problems if applied to the actual experimental situation.

The molecular basis for the highly aberrant behaviour of K9 and its derivatives in our modified system is not known. This keratin peptide is unusual also in having a considerably higher molecular weight than other type-I cytokeratins (13).

Because of the anatomy of the transition zone between living and cornified epidermis, the experimental design used did not permit an exact determination of the tissue site at which each modification step takes place. From the results it seems conceivable however, that K1 as well as K9 both undergo their first modification step at the same site and that this site is close to where the initiation of cornification takes place. This would mean that the second modifications, although occurring at different levels for the two polypeptides, take place in the stratum corneum and represent some form of 'maturation', maybe stabilization, of the keratin fibres. The second modification steps for the two polypeptides differ also in the extent to which they are completed within the stratum corneum. This suggests that each step might be separately regulated. The constant manner in which the modification steps occur in individuals with normal skin suggests that they may have important roles in the keratinization process (5) and in the maintenance of a normal stratum corneum function.

It also seems conceivable that the different modification steps are produced by proteolytic cleavages. If this is true, considerable amounts of low-molecular weight polypeptides should be formed in the different steps. Since the later steps occur within the stratum corneum they may be significant sources of osmotically active molecules, important for the functioning of the stratum corneum.

As demonstrated by two-dimensional electrophoresis (cf. Fig. 1) the net electrical charges of K1 and K9 become more negative as a result of the modification steps (i.e. the modifications leads to apparent decreases in isoelectric pH-values for the two polypeptides). The most pronounced changes in this respect occur within the stratum corneum. This may be of importance, for instance in changing the capacity of keratin filaments close to the skin surface to bind metal ions.

It is also tempting to speculate on possible regulatory functions of, e.g., diffusible polypeptides that may be formed in the conversion K9a->K9b in the superficial layers of stratum corneum.

The differences between the modification steps may have a variety of explanations. They could be exerted by different enzymes that are activated in different phases of the keratinization process, the polypeptides themselves could differ in their susceptibility to proteolysis, or the various keratin polypep-

tides could be associated with different non-keratinous molecules, e.g. filaggrins (4), in such a way as to give them different degrees of protection from proteolysis.

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