Biochimica et Biophysica Acta, 657 (1981) 268—276

BBA 69188

RAT HAIR FOLLICLE AND EPIDERMAL TRANSGLUTAMINASES

BIOCHEMICAL AND IMMUNOCHEMICAL ISOENZYMES

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(Received August 4th, 1980)

Key words: Transglutaminase isoenzyme; Localization; (Rat skin, Hair follicle)

Summary

Epidermal and hair follicle transglutaminases (1,4-α-D-glucan: orthophosphate α-D-glucosyltransferase EC 2.4.1.1) were differentially isolated and subsequently purified from newborn or 4—5-day-old rats. Both enzymes migrated identically on ion-exchange chromatography but were widely separated by block electrophoresis, with the epidermal enzyme migrating further toward the anode. Each enzyme was finally purified by gel filtration. Epidermal transglutaminase had an apparent molecular weight of 56 000—58 000 in this medium and in gels containing sodium dodecyl sulfate (SDS), while hair follicle transglutaminase had a molecular weight of 52 000—54 000 and was reduced to two apparently identical subunits of a molecular weight of 27 000 by denaturing media.

Antiserum specific to each transglutaminase was produced in chickens; when conjugated to fluorescein these antisera localized the enzymes to the granular layer of epidermis and the inner root sheath of hair follicles, respectively.

Introduction

The transglutaminase enzymes mediate covalent intermolecular ε(γ-glutamyl) lysine crosslinking between structural proteins, such as fibrin or fibronectin. Such crosslinking stabilizes the fibrin clot and facilitates healing of wounds [1]. Transglutaminases have been isolated from surface epidermis of bovine snout and human skin [2,3] and also from hair follicle tissue [4—6]. The function of this enzyme in epidermis is believed to be the stabilization of proteins lining cell membranes of keratinized tissues, lending stability, strength and resistance to denaturation to the skin surface.

Recent evidence [7—9] indicates that transglutaminase enzymes from surface epidermis and hair follicle tissue may be immunochemically distinct. Surface epidermal transglutaminase and hair follicle transglutaminase have never before been differentially isolated from the same animal species; this has prevented close comparative analysis of the two enzymes as isoenzymes. Distinction between these two enzymes may have clinical relevance, in that many disorders of keratinization involve surface epidermis without affecting hair follicle structures (for example, psoriasis) and vice versa. Knowledge of subtle differences between keratinization processes in these two tissues is, therefore, of vital importance.

We have previously reported that epidermal transglutaminase is continuously produced in surface epidermis, which has a constant growth rate, while hair follicle transglutaminase is synthesized only during the anagen, or growth phase, of hair follicles [10].

In longitudinal quantitative studies of transglutaminase activity in perinatal rats, two serial rises in enzyme activity were shown biochemically and histochemically: one at gestational day 17, corresponding to the onset of terminal keratinization in surface epidermis, and the second at postnatal day 4—5, corresponding to the onset of the first anagen phase in hair follicles [10].

Since the first hair cycle in these animals is synchronous over the entire pelage, a maximal yield of both epidermal and hair follicle transglutaminase should be possible at postnatal day 4—5.

We now report the differential purification, characterization and immunochemical localization of epidermal and hair follicle transglutaminase from day 4—5 rat epidermal extracts.

Materials and Methods

Coomassie brilliant blue G-250 was obtained from Spectrum (Los Angeles, CA) and used exclusively for protein determinations [9]. Transglutaminase was assayed by measuring the incorporation of dansyl cadaverine into casein, as previously described [2], and reported as relative fluorescence against a blank containing all components of the reaction except dansyl cadaverine.

Enzyme purification

Simonson rats (Gilroy, CA) were killed by decapitation and the trunk epidermis was removed from 150 newborn or 4—5-day-old animals by a 30 s immersion of the whole skin in a 56°C water bath. Epidermal fragments were then snap-frozen and pulverized in liquid nitrogen, and the resulting powder was homogenized in a Polytron (Brinkman Instruments, Westburg, NY) with 130 ml cold 0.025 M ammonium acetate/0.001 M EDTA, pH 8.5 (starting buffer). Following centrifugation at 40 000 × g for 45 min in a Sorvall RC2B refrigerated centrifuge, the supernatant was applied to a 2.5 × 50 cm column of DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden). This and all subsequent steps were conducted at 4°C. After washing with starting buffer, the column was developed with a 1000 ml linear gradient to 0.3 M NaCl in starting buffer. The conductivity of eluted fractions was monitored and protein was expressed as A_{280nm} in a Zeiss PMQ II spectrophotometer with a 1 cm light path. Specific

activity, calculated at each step of protein purification, was expressed as amine incorporating units (AIU) per mg of protein/h [2].

Fractions containing enzyme activity were concentrated to 5 ml in an Amicon ultrafiltration apparatus (Amicon Corp., Lexington, MA) with a PM-10 filter, dialyzed and subjected to block electrophoresis in Pevikon (Mercer Chemical Corp., Amityville, NY) as previously described [2], in a 0.025 M ammonium acetate/0.001 M EDTA buffer at pH 8.6.

The two recovered enzyme-containing peaks were separately concentrated to 1.5 ml and passed through a 0.9 × 90 cm column of ACA-44 Ultrogel (LKB Western Instruments, Inc., Pleasant Hill, CA) which had been equilibrated with starting buffer and pre-calibrated with Blue Dextran, human IgG, bovine serum albumin and soybean trypsin inhibitor. Flow was maintained at 5 ml/h.

Acrylamide gel electrophoresis was performed according to a modification of the method of Shapiro et al. [12]. Protein samples were boiled in 1% SDS with or without 1% β-mercaptomethanol at 100°C for 10 min prior to application and their migration compared with that of standard proteins of known molecular weight.

Immunochemical studies

Purified rat epidermal or hair follicle transglutaminase was emulsified 1 : 1 with complete Fruend's adjuvant and a volume containing 0.03 mg specific antigen was injected intramuscularly into young male chickens. An identical booster injection was given two weeks later. One week after the second injection, blood was withdrawn from wing veins and the serum was separated and stored at −20°C. Ouchterlony plates were prepared with standard agar containing 1.5 M NaCl [13], which served to enhance precipitation of chicken antibody complexes.

Punch biopsies (4 mm) of 4—5-day-old rat skins were sectioned at 5 μm in a cryostat at −20°C, freeze-dried and paraformaldehyde vapor-fixed [9], and individual sections were placed within wax rings on microscopic slides for staining.

Specific antibody-containing and preimmune sera were enriched in gamma globulin by 50% cold neutral $(NH_4)_2SO_4$ precipitation, dialyzed exhaustively, and directly conjugated to fluorescein isothiocyanate by an established procedure [14].

Freeze-dried 5 μm tissue sections were incubated with fluorescein-conjugated sera for 5—30 min, then washed for 60 min 0.01 M Tris/0.15 M NaCl, pH 7.5. For comparison, 3 mm punch biopsy specimens were fixed overnight in 10% formalin, sectioned at 5 μm in a freezing microtome and directly stained with fluorescein-conjugated antisera.

Results

Heat separation of rat epidermis yielded predominantly surface epidermis in newborn, and epidermis plus hair follicles in 4—5-day-old rats (Fig. 1a and b). Day 4—5 animals were used for purification of transglutaminase isoenzymes.

A single peak of transglutaminase activity was evident after DEAE-Sepharose ion-exchange chromatography of day 4—5 extracts, eluting at 0.17 M NaCl

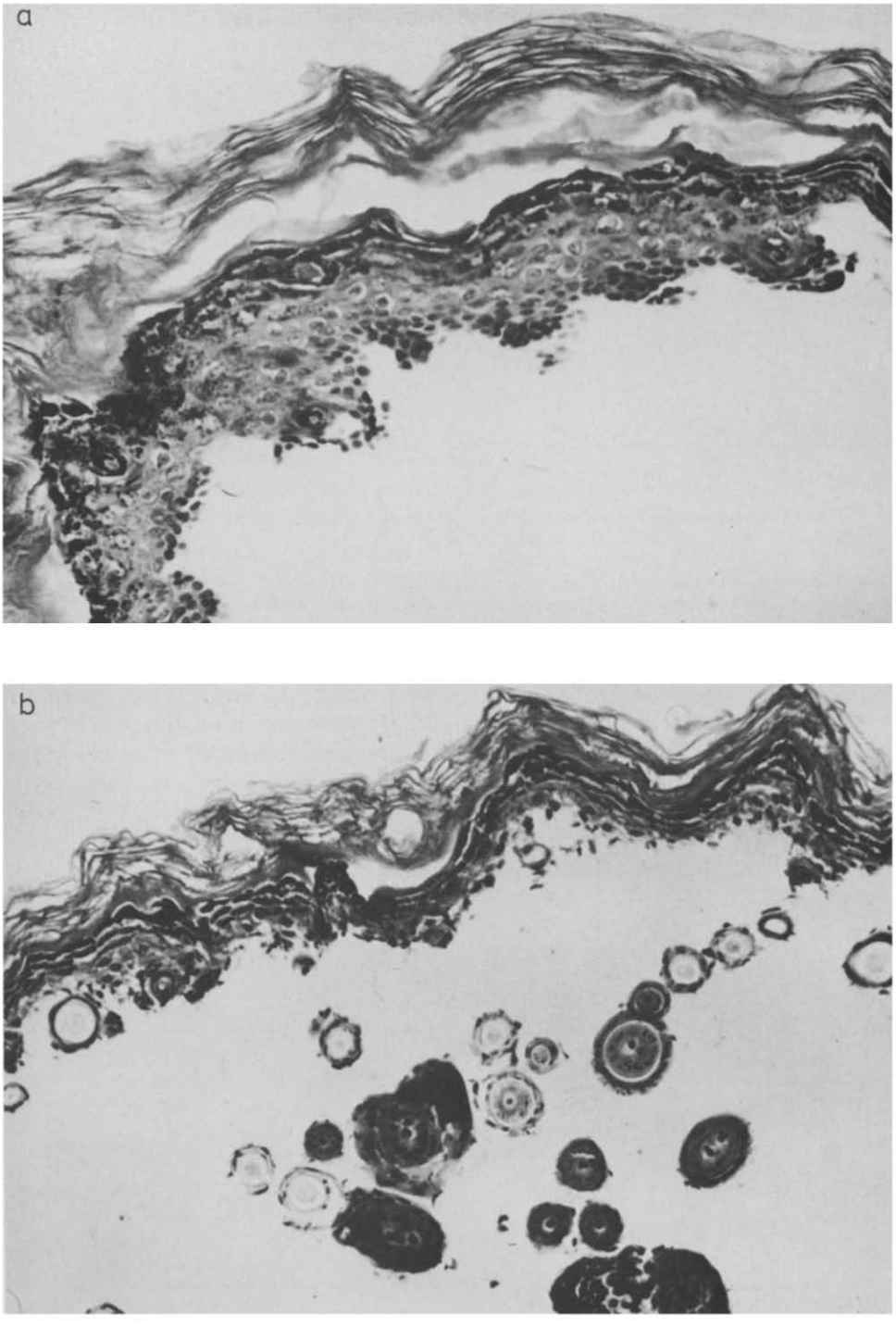

Fig. 1. Comparison of newborn (Day 0) and Day 4—5 heat-separated rat epidermis. (a) Day 0 epidermis shows small follicular buds protruding from the basal layer of the epidermis (×234). (b) Day 4—5 epidermis shows numerous follicular structures in various stages of differentiation (×234).

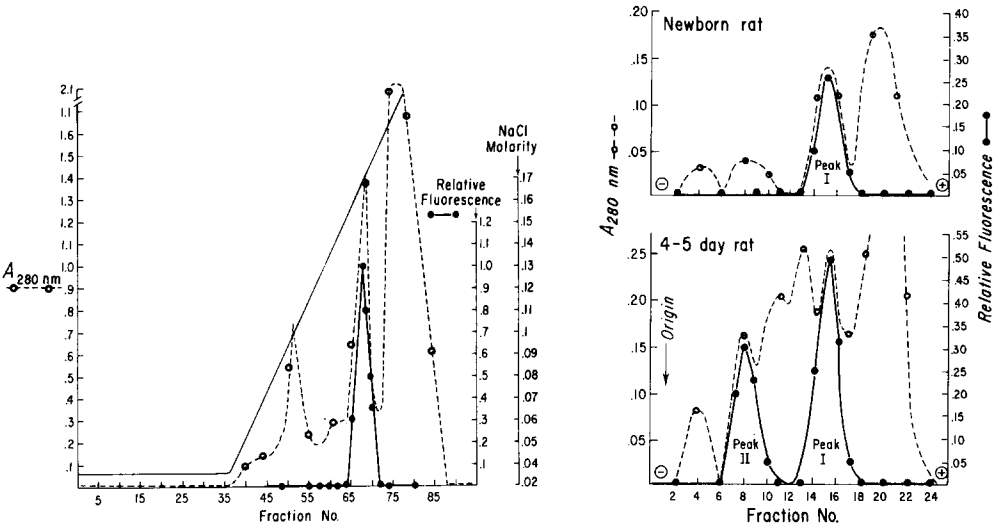

Fig. 2. DEAE-Sepharose CL-6B ion-exchange chromatography. Transglutaminase elutes as a single peak at 0.1 M NaCl.

Fig. 3. Pevikon electrophoresis of enzyme-containing fractions from the ion-exchange chromatography step. Partially purified newborn enzyme (above) is a single species (peak I) while the Day 4—5 material (below) contains two distinct enzymes (peak I, peak II).

(Fig. 2). When fractions from this peak were combined and electrophoresed in Pevikon, however, 2 enzyme peaks (I and II) were apparent. Pevikon electrophoresis of the enzyme peak obtained after identical treatment of newborn epidermal extracts yielded only one of the two peaks, Peak I (Fig. 3). This enzyme was more electro-negative and migrated farther toward the anode.

Separate ACA-44 Ultrogel filtration of peak I and peak II transglutaminases (Fig. 4) showed that the peak II enzyme had a lower apparent molecular weight

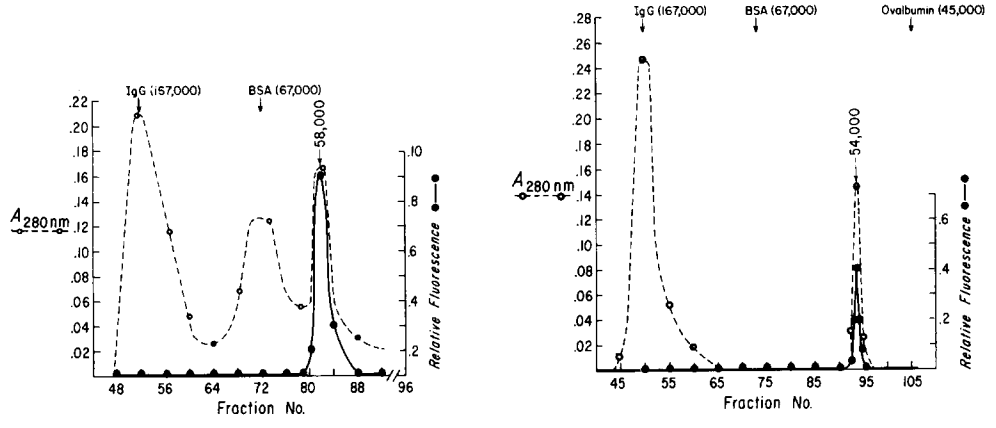

Fig. 4. ACA-44 Ultrogel filtration of enzymes from Pevikon electrophoresis. Column was pre-calibrated with Blue Dextran, human IgG, bovine serum albumin (BSA) and soybean trypsin inhibitor. (a) peak I enzyme has an M_r of 58 000. (b) peak II enzyme has an M_r of 54 000.

TABLE I

PURIFICATION OF RAT EPIDERMAL AND HAIR FOLLICLE TRANSGLUTAMINASES

ATU, amine incorporating units.

Purification step	Protein (mg)	AIU	AIU/mg	Purification	Yield (%)
Crude homogenate	112.5	560	4.91	1.0	100
DEAE-Sepharose	13.0	240	18.5	3.8	42
Pevikon					
peak 1	0.5	20	40	8.1	12.5
peak 2	1.0	50	50	10.2	
ACA-44 Ultrogel					
peak 1	0.05	8	160	32.6	5.9
peak 2	0.08	25	312.5	63.6	

(52 000—54 000) while the peak I enzyme had a molecular weight of 56 000—58 000. The final yield of the two enzymes together was 5.9%, as shown in Table I.

After electrophoresis in SDS-containing gels, purified peak I transglutaminase formed a single band at M_r = 58 000 by comparison with protein standards, while the peak II enzyme was reduced to subunits of M_r = 27 000 (Fig. 5). Identical results were obtained with and without mercaptoethanol. Retrospectively, comparative SDS-containing gels were performed using the enzyme-containing peak recovered from DEAE-Sepharose chromatography of newborn and 4—5 day epidermal extracts. A visible band at M_r = 58 000 in the enzyme peak from both ages was seen but an additional band at 27 000 was present only in the latter (results not shown).

Enzyme-specific antisera raised in chickens precipitated in agar with their respective antigens, as shown in Fig. 6a and b. Peak I transglutaminase was immunochemically detectable in both newborn and 4—5 day crude epidermal

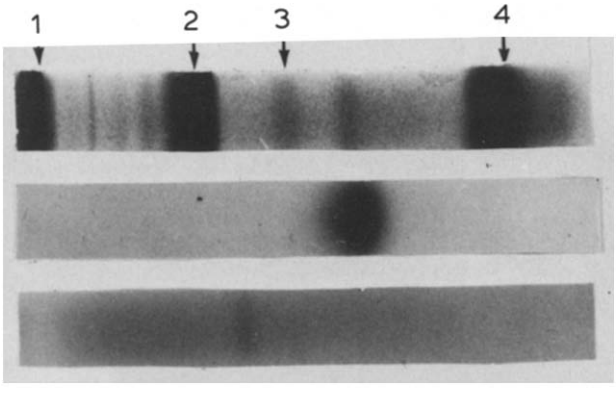

Fig. 5. SDS-containing gels of purified epidermal and hair follicle transglutaminase. Epidermal transglutaminase (bottom) has a molecular weight of 58 000, while hair follicle transglutaminase (center) has a molecular weight of 27 000. The gel above contains standard proteins: 1, γ-globulin (160 000); 2, bovine serum albumin (67 000); 3, ovalbumin (45 000); 4, cytochrome *c* (12 528).

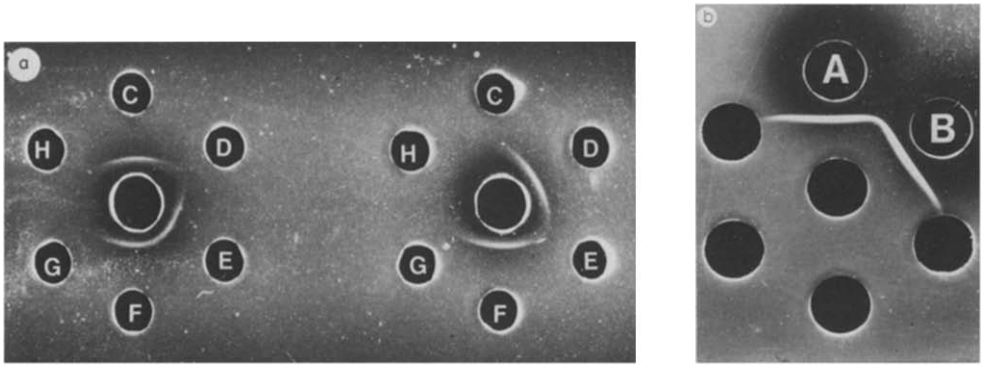

Fig. 6. Ouchterlony double-diffusion analysis of chicken antibodies to epidermal and hair follicle transglutaminases. (a) Center well, left: antiserum to peak I (epidermal) transglutaminase: Center, right, antiserum to peak II (hair follicle) transglutaminase. Clockwise, from top: C, purified epidermal transglutaminase; D, purified hair follicle transglutaminase; E, newborn epidermal extract; F, Day 4—5 extract; G, bovine plasma transglutaminase. (b) Center well, antiserum to epidermal transglutaminase. A. Purified epidermal transglutaminase; B. Day 4—5 extract. Similar lines of identity were present with purified hair follicle tranglutaminase and Day 4—5 extract.

extracts but peak II transglutaminase only in 4—5 day extracts. No cross-reactivity in agar was noted between antisera. No spurs were apparent when purified enzyme and crude extract were placed in adjacent wells (Fig. 6b).

Incubation of freeze-dried, paraformaldehyde-fixed tissue sections with antiserum specific for peak I transglutaminase resulted in development of intense fluorescence along the epidermal-stratum corneum border, with no staining of hair structures below the follicular infundibulum (Fig. 7a). In Fig. 7b, fluorescein-conjugated antiserum to hair follicle transglutaminase is seen to label the medulla and inner root sheath of the follicles. This antiserum failed to label epidermis. Preimmune serum similarly conjugated did not label either the epidermis or its appendages.

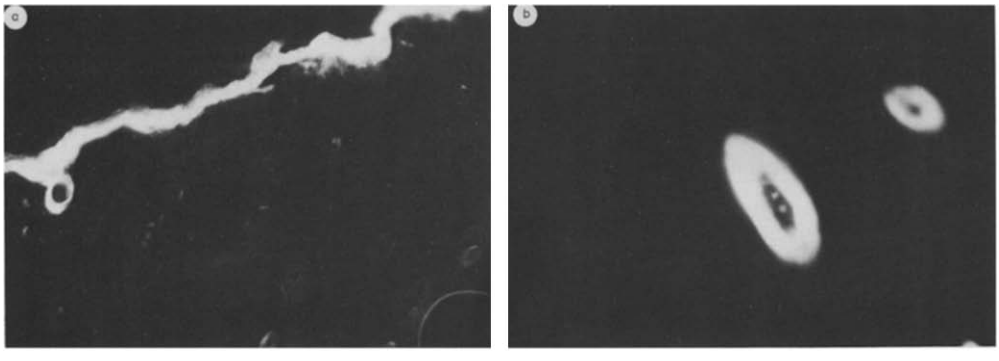

Fig. 7. Immunochemical localization of epidermal transglutaminase and hair follicle transglutaminase. (a) Day 4—5 epidermis, incubated with fluorescein-conjugated chicken antiserum to rat epidermal transglutaminase. Staining is seen to be confined to the granular layer of the epidermis. Hair follicles, in the malpignian layer in lower portion of photograph are negative (X200). (b) Day 4—5 epidermis, incubated with fluorescein-conjugated antiserum to rat hair follicle transglutaminase. Positive reaction is predominantly seen in cells of the inner sheath, and also is present in medullary cells, photograph taken from cross-section of malpighian layer (X375).

Absorption of the antisera with specific enzyme antigen prior to incubation with tissue sections abrogated all fluorescence.

Routine formalin-fixation was inadequate to retain enzyme antigen in situ for antibody labeling.

Discussion

The existence of immunochemical isoenzymes of transglutaminase in epidermis and hair follicles was anticipated in 1976, when it was found that antiserum to bovine snout epidermal transglutaminase failed to precipitate in agar with, or to functionally inhibit, transglutaminase in hair follicle extracts [7]. These observations were substantiated by the discovery that fluorescein-conjugated antiserum to bovine epidermal transglutaminase stained epidermis but not follicles [9]. Similar results were reported with human epidermal transglutaminase antisera [8]. Biochemical differences between the two enzymes were suggested by the findings of Chung and Folk [6], that purified guinea pig hair follicle transglutaminase had a molecular weight of 56 000 on molecular sieve chromatography but was reduced to subunits of 27 000 by SDS. These investigators proposed a structural model containing two identical subunits for hair follicle transglutaminase. Subunits have not been found in epidermal transglutaminase from any species.

Differences between the two enzymes have been directly confirmed by the present study, which has shown that different isozymic forms of transglutaminase are indeed present in epidermis and in epidermal appendages of the same species. The individual isozymes are manufactured at different times during skin maturation; only the surface enzyme is present in large enough quantities to be immunochemically or biochemically detectable, while the follicular enzyme approaches maximal activity postnatally. Since the two enzymes are clearly separable in 4—5-day-old animals by block electrophoresis, minute, biochemically undetectable amounts of hair follicle transglutaminase present in extracts of newborn epidermis would be eliminated following this purification step.

It is probable that the natural substrates for the two enzymes are not identical. Although the initial substrate for hair follicle transglutaminase has not been isolated, tryptic peptides of fractions of hair follicle extracts have been found to contain both ϵ-(γ-glutamyl) lysine bonds and the unusual amino acid citrulline [4]. Interestingly, a substrate for bovine epidermal transglutaminase isolated in our laboratory, which has been more completely characterized, has now been shown to contain citrulline [15,16] and to have an amino acid content similar to that described by Matoltsy for the membrane protein of epidermal cells [17]. Labelled antisera to tryptic peptides, containing citrulline of hair follicle stain inner-root sheath and medulla cells in a pattern coincidental with trichohyalin granules [18]; similar immunofluorescent localization of epidermal transglutaminase substrate shows cytoplasmic distribution in malpighian cells, and inner cell membrane concentration in upper granular and stratum corneum cells [16].

Further biochemical studies, including amino acid sequencing and kinetic studies using antibodies to the two enzymes of the two molecules are necessary

to elucidate the exact relationship of these two unique epidermally-derived isoenzymes.

Acknowledgements

The authors acknowledge the technical assistance of Dr. H. Uno, Ms. C. Lobitz and Ms. R. Milbeck and the editorial advice of Dr. K. Wuepper. Supported, in part, by National Institutes of Health Grant AM 17687. L.L.P. was a recipient of a 1979 Syntex Summer Fellowship. M.M.B. was the recipient of Clinical Investigator Award No. AM 00391 from the National Institutes of Health.

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