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ENDOGENOUS SUBSTRATES FOR EPIDERMAL TRANSGLUTAMINASE *

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Summary

Potential in vivo substrates for epidermal transglutaminase have been isolated and partially characterized in human stratum corneum and new born rat epidermis. [^{14}C]Putrescine and dansylcadaverine were incorporated into epidermal proteins in vitro. Two high molecular weight proteins incorporated the labels in both the rat and human homogenates. One of the proteins was too large to enter a 4% sodium dodecyl sulfate-polyacrylamide spacer gel; the other was seen at the interface between the spacer gel and a 10% sodium dodecyl sulphate-polyacrylamide running gel. These proteins were present in a buffer extract, sodium dodecyl sulphate-dithiothreitol extract and NaOH extract. The labels were also incorporated into protein in the insoluble pellet remaining after the afore-mentioned extractions. The incorporation of putrescine and dansylcadaverine was time dependent, and was inhibited by known inhibitors of epidermal transglutaminase. The two high molecular weight proteins had similar amino acid composition, characterized by high glycine, glutamic acid, serine and aspartic acid. Their amino acid composition was similar to, although not identical with, the amino acid composition of α-keratin proteins.

Epidermal homogenates incubated in the presence of transglutaminase showed progressive insolubilization of the protein. This cross-linking was inhibited by putrescine.

[^{14}C]Glycine, [^{14}C]histidine and [^{14}C]proline were incorporated into epidermal proteins in newborn rats in vivo. The glycine-labelled protein became progressively more insoluble when incubated in vitro in the presence of transglutaminase. In vitro incubation with transglutaminase had no effect on the histidine- and proline-labelled proteins.

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Introduction

Transglutaminases are present in a number of mammals, and have been isolated and characterized from human epidermis [1,2] and cow epidermis [3]. The enzyme is also present in the hair follicles of a variety of species including man [4], cow [5], sheep [6], rats [6] and guinea pigs [6,7]. It is clear that the hair follicle transglutaminase has as its primary substrate a citrulline-rich protein which is present both in the medulla and in the internal root sheath of the hair [8]. The substrate for epidermal transglutaminase is still undetermined. Identifying the endogenous epidermal substrate is important for ascertaining the physiological role of the ϵ-(γ-glutamyl)lysine cross-link in epidermis.

There have been several approaches to the identification of proteins which are cross-linked or modified by epidermal transglutaminases. In one study, epidermal proteins were isolated, and the blocked ϵ-amino groups quantitated [9]. There were no blocked ϵ-amino groups in the urea-soluble fractions of cow snout epidermis, but blocked ϵ-amino groups were present in the urea-sulfhydryl-soluble proteins of the cow snout. Similarly, in human stratum corneum, blocked ϵ-amino groups were found in urea-sulfhydryl-soluble proteins by cyanoethylation, and further studies revealed the presence ϵ-(γ-glutamyl)lysine cross-links [10] in these proteins. The protein fraction containing ϵ-(γ-glutamyl)lysine cross-links, however, was heterogeneous. The present studies were done to determine which proteins in human stratum corneum and in newborn rat epidermis were cross-linked by a transglutaminase-dependent mechanism. To perform these studies, the ϵ-glycine analogues putrescine and dansylcadaverine were incorporated into proteins which were potential substrates. In addition, the progressive cross-linking and insolubilization of epidermal proteins, and the effect of inhibiting that cross-linking were studied.

Materials

Adult CD rats were purchased from Charles River Labs. (Wilmington, Mass.). The animals were maintained on a Purina Rat Chow diet, and bred in the lab. Common chemicals were reagent grade. Suppliers of other materials included: [U-^{14}C]glycine (specific activity, 96.0 Ci/mol), [U-^{14}C]proline (specific activity, 25.4 Ci/mol), [U-^{14}C]histidine (specific activity, 282 Ci/mol), [1,4-^{14}C]-putrescine (specific activity, 51.8 and 72.6 Ci/mol), Aquasol and Protosol from New England Nuclear (Boston, Mass.); dansylcadaverine, Cyclo Lab. (Los Angeles, Calif.) or Sigma (St. Louis, Mo.); Trypsin (Type III), bovine serum albumin, α-chymotrypsinogen and cytochrome *c*, Sigma; 1.5 M, 15 M and 150 M agarose, sodium dodecyl sulfate (SDS) and reagents for polyacrylamide gels, Bio-Rad (Richmond, Calif.); dithiothreitol, Bachem Feinchemikalien (Switzerland); GF/C filters, Whatman (Clifton, N.Y.); RP Royal X-omat X-ray film, Kodak (Rochester, N.Y.).

Methods

Dansylcadaverine incorporation into human stratum corneum. 5 ml 0.1 M glycine/NaOH, pH 9.5, was added to six flasks, each containing 100 mg of

scales from a patient with lamellar ichthyosis. The scales were homogenized with a Duall 24 glass-glass homogenizer. 2 mM dithiothreitol/10 mM $CaCl_2$/2 mM dansylcadaverine (final concentrations) were added to five of the flasks. The sixth flask, which served as a control, contained 20 mM iodoacetamide/20 mM EDTA/2 mM dansylcadaverine (final concentrations). The flasks were incubated at 37°C in a shaking water bath for various time periods; 35 min, 1, 2, 4, 18 h; the control flask was incubated for 18 h. At the end of the incubation period, the material in each flask was centrifuged 12 000 × *g* for 10 min at 4°C and the supernatants were removed. The pellets were extracted once in 100 mM Tris (pH 9.0)/1% SDS/50 mM dithiothreitol at 4°C for 24 h, centrifuged as above, and then were extracted twice in 0.1 M NaOH at 4°C for 24 h each. Portions of all extracts were electrophoresed on SDS-polyacrylamide gels; the remaining portions of the extracts and final pellets were dialyzed exhaustively against distilled water, lyophilized and weighed. In similar experiments to determine the effect of 0.05—50 mM putrescine on the in vitro cross-linking of stratum corneum proteins, flasks containing putrescine/2 mM dithiothreitol/10 mM $CaCl_2$ in buffer were incubated for 18 h 37°C and then treated as described above.

Dansylcadaverine incorporation into newborn rat skin in vitro. 30 newborn rats (all less than 24 h old) were killed by decapitation and their skins removed. Each skin was placed in water at 56°C for 30 s, then placed on ice and the epidermis was peeled from the dermis. 1.3 g (wet weight) of epidermis was homogenized in 30 ml 0.1 M glycine/NaOH (pH 9.5) at 4°C, using a Duall 24 glass-glass homogenizer. To flasks 1 and 2, $CaCl_2$ and dithiothreitol were added to 10 and 2 mM, respectively. 2 mM dansylcadaverine (final concentration) was added to flask 2. Flask 3, which served as a control, contained 20 mM EDTA/20 mM iodoacetamide. The volume in all flasks was brought to 40 ml with the glycine/NaOH buffer. The flasks were incubated at 37°C in a shaking water bath for 18 h at which time buffer, SDS, and NaOH extracts, and the final extracts were prepared as previously described.

Incorporation of [^{14}C]putrescine into newborn rat epidermis in vitro. Newborn rat epidermis was suspended at 80 mg/ml in 35 ml 0.111 M glycine/NaOH buffer (pH 9.5)/0.55 mM EDTA/11 mM $CaCl_2$ and homogenized. 10 μCi [^{14}C]-putrescine was added and the mixture incubated at 37°C in a shaking water bath, overnight. The homogenate was centrifuged at 12 000 × *g* for 10 min at 4°C. A 100 μl aliquot of the supernatant was dissolved in 5 ml of Aquasol, and another 100 μl aliquot was precipitated with 1 ml of 5% trichloroacetic acid, and filtered through a GF/C Whatman Filter and placed in 10 ml of Aquasol. The remainder of the supernatant (34.8 ml) was concentrated to 10 ml with an Amicon (PM-10 filter) concentrator at 4°C. 1% SDS and 50 mM dithiothreitol (final concentrations) were added to the concentrated material. The solution was boiled 20 min and chromatographed on a 1.5 M agarose column under the conditions described below. The pellet from the incubation mixture was extracted first in 20 ml 2% SDS/50 mM dithiothreitol/100 mM Tris (pH 8.7) then with 0.1 M NaOH as described above. The SDS extract was boiled for 10 min, then put on a 1.5 M agarose column and treated as described above. The NaOH extract and final pellet were dialyzed and treated as above.

Incorporation of ^{14}C-labelled amino acids into epidermal protein from new-

born rat skin: a combined in vivo and in vitro method. 20—40 newborn rats (less than 3 days old) were injected subcutaneously with 1 μCi of the ^{14}C-labelled amino acid (glycine, proline or histidine) dissolved in 0.1 ml saline. 70 min after the injections, the animals were killed by decapitation and the epidermis removed by heating. The epidermis was suspended in 0.1 M glycine/NaOH buffer (pH 9.5) at 40 mg/ml (wet weight/v), then homogenized at 4°C with a Duall 24 glass-glass homogenizer. The homogenate was equally divided into five flasks and 10 mM $CaCl_2$ and 2 mM dithiothreitol (final concentrations) were added to four of the flasks. The fifth flask contained 10 mM EDTA/20 mM iodoacetamide. Additional glycine/NaOH buffer was added to bring the final concentration of protein in each flask to 30 mg/ml. The first flask (T_0) was kept on ice and centrifuged immediately. The other flasks were incubated in a shaking water bath at 37°C, and removed at 3 h, 6 h and 18 h. The EDTA/iodoacetamide control was incubated for 18 h at 37°C. After removal from the water bath, the contents of each flask were centrifuged 12 000 $\times g$ for 10 min. Supernatants and final pellet were prepared as described above. The radioactivity of the lyophilized proteins were determined by Protosol digestion and scintillation counting. 1 mg of protein was suspended in 0.2 ml distilled water/1 ml protosol, then incubated overnight at 37°C. The solutions were neutralized with 1 M HCl and 10 ml Aquasol was added to each.

SDS-agarose chromatography. 20 mg of the lyophilized extracts were dissolved in 10 mM Tris (pH 9.0)/2% SDS/10 mM dithiothreitol. The material was incubated at 50°C for 1.5 h, then applied to a 1.5 M agarose (2.5 × 100 cm) or a 15 M agarose (1.5 × 50 cm), or a 150 M agarose (2.5 × 23 cm) column. The column buffer contained 100 mM Tris (pH 9.5)/0.1% SDS/1 mM EDTA/0.15 M NaCl. The sample was chromatographed at 25°C. Samples on the 1.5 M and 15 M agarose column were eluted at a rate of 16 ml/h. The 150 M agarose column was run under gravity-reverse flow (1.0-ml fractions). Material eluted from the column was monitored at 280 nM with a Uvicord II column monitor. Aliquots of selected fractions were monitored in Aquasol for radioactivity, or analyzed under long wave ultraviolet light for fluorescence, then electrophoresed on SDS-polyacrylamide gels. Fractions were pooled, dialyzed against distilled water, lyophilized and re-electrophoresed. Lyophilized material was hydrolyzed under N_2 and analyzed on a Beckman amino acid analyzer as previously described [11].

Trypsin digestion of high molecular weight protein from rat epidermis. The samples were neutral buffer and SDS extracts of newborn rat epidermis which had been labelled with dansylcadaverine or [^{14}C]putrescine. The proteins were fractionated on 1.5 M agarose column in order to separate the "very high" and "high" molecular weight proteins (see below). The column fractions were dialyzed against deionized water and lyophilized. The lyophilized material was dissolved in 0.5 M Tris (pH 8.5) at 2 mg/ml at 4°C. A 100 μl aliquot was removed to be used as the control sample. An aliquot of trypsin dissolved in 0.001 M HCl was added to the substrate, such that the final trypsin concentration was 10 μg/ml. Immediately after the addition of trypsin another 100 μl aliquot was taken and called a "10 s sample". The flask with the digestion solution was put into a shaking water bath at 37°C for 6 h, followed by an additional incubation at 25°C for a further 12 h. At various times, 100-μl samples

were taken from the trypsin solution. 50 mM dithiothreitol and 1% SDS were added to the 100-μl aliquots as they were removed from the flask, and the aliquots were immediately boiled for 5—10 min. The samples were run on SDS-polyacrylamide gels [12]. Gels which contained radioactively labelled samples were dried and autoradiographed by fluorography [13] using RP Royal X-omat film developed in an automatic X-omat developer. The carbohydrate content of the proteins was determined by a phenol-H_2SO_4 assay described by Hirs [14] using glucose as a standard.

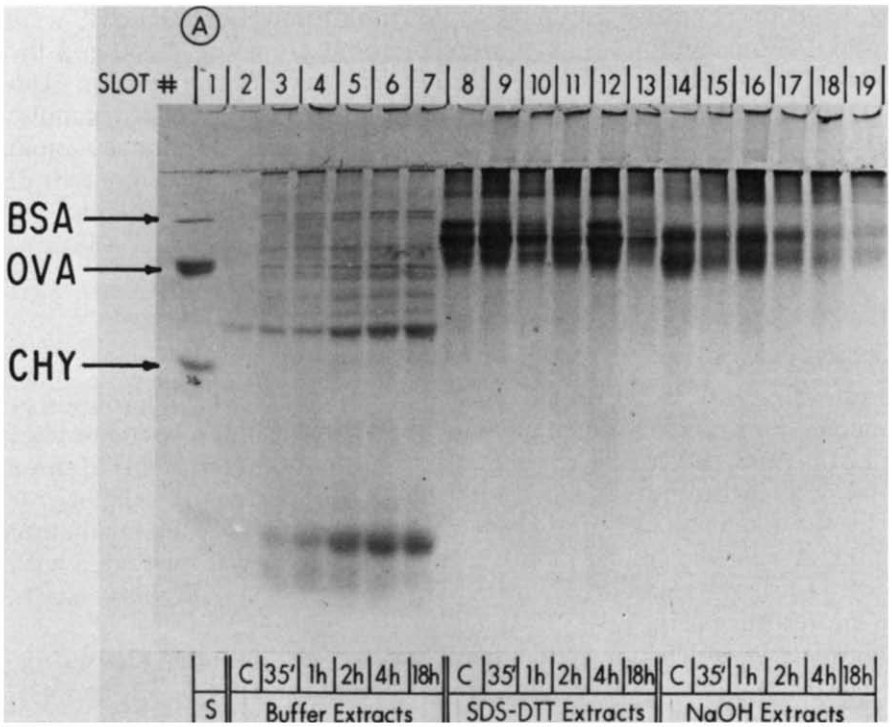
Results

Dansylcadaverine incorporation into human stratum corneum

Dansylcadaverine was incorporated into high molecular weight proteins of the stratum corneum. These labelled proteins were present in the buffer extract as well as in the SDS and NaOH extracts. The multiple other proteins in these extracts did not incorporate dansylcadaverine. The degree of labelling as measured by the intensity of fluorescence increased with increasing incubation times (Fig. 1). A control sample, in which the homogenate was incubated with inhibitor of transglutaminase (iodoacetamide and EDTA) had only barely detectable fluorescence (Fig. 1).

In the extracts in which transglutaminase activity was inhibited the buffer, SDS, and NaOH extracts, and the final pellet contained 18, 65, 13, and 4%, respectively, of the total weight. In the extract incubated for 18 h in the presence of active transglutaminase ($CaCl_2$ and dithiothreitol present) similar extracts contained 6, 41, 25 and 28%, respectively, of the total weight. Incubation for 30 min, 1, 2, and 4 h gave values between these two extremes. When the 18 h incubation with $CaCl_2$ and dithiothreitol was performed in the presence of 0.05—50 mM putrescine, 3 mM putrescine inhibited by two-thirds the increase in the amount of weight present in the final pellet; but 50 mM putrescine was no more effective than 3 mM putrescine. The SDS extract of the homogenates incubated with 1—3 mM putrescine when examined by SDS electrophoresis showed more of the 50 000—60 000 dalton protein than controls.

The SDS extract was chromatographed on 1.5 M and 15 M agarose columns. The chromatography of the 15 M agarose columns showed two distinct protein peaks. The first peak contained only "high" and "very high" molecular weight protein ("very high" refers to material which would not enter the 4% polyacrylamide spacer gel; "high" refers to material which remained at the interface between the spacer gel and the 10% running gel). When run on SDS-polyacrylamide gels, "high" and "very high" molecular weight bands were fluorescent and stained with Coomassie Blue. No other protein bands were present in this peak. The protein from the first peak was hydrolyzed and its amino acid content determined (Table I). Fractions from the second major protein peak contained lower molecular weight proteins, some of which contained extremely faint fluorescence. The chromatography of the SDS extract in a 1.5 M agarose column showed five major protein peaks (Fig. 2). The first two peaks contained "high" molecular weight proteins and "very high" molecular weight proteins, both of which were fluorescent, as well as a small amount of fluorescent protein of 50 000 daltons. A large amount of non-fluorescent protein eluted in the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BSA
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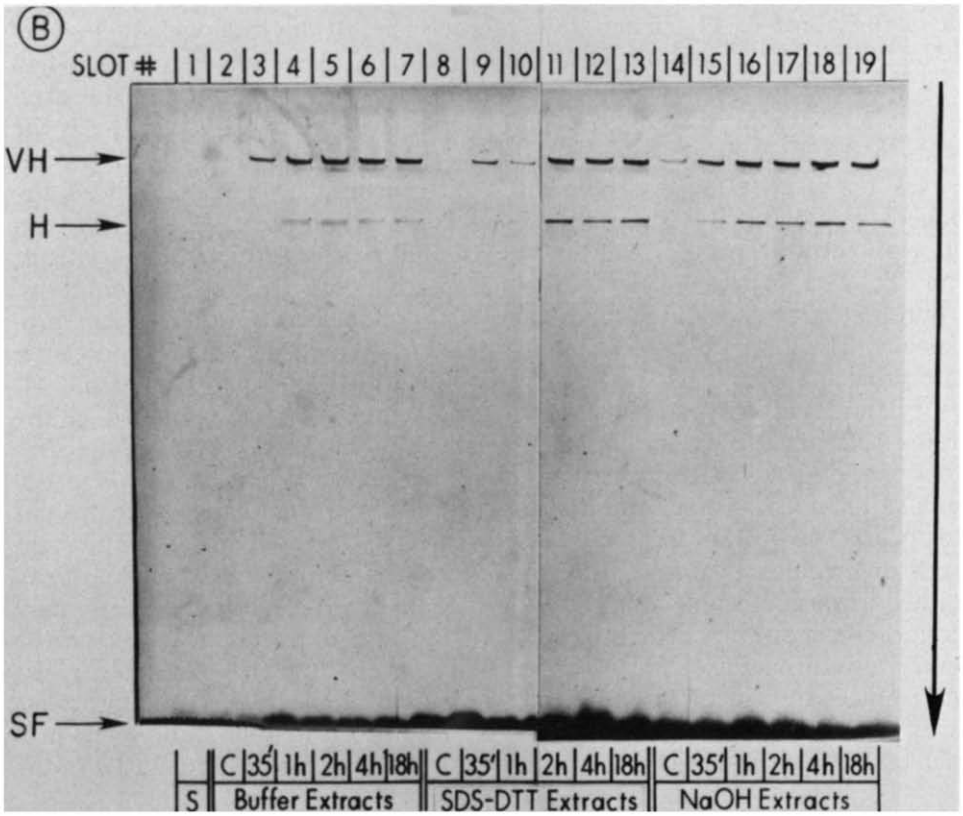
Buffer Extracts
SDS-DTT Extracts
NaOH Extracts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Buffer Extracts
SDS-DTT Extracts
NaOH Extracts

last three peaks. In addition, a portion of the extract did not enter the column and was seen on top of the column as a fluorescent band with long wave ultraviolet light. The protein which did not enter the column when electrophoresed, contained "high" and "very high" molecular weight proteins. The "high" and "very high" molecular weight proteins contained 5% carbohydrates by phenol-H_2SO_4 determination.

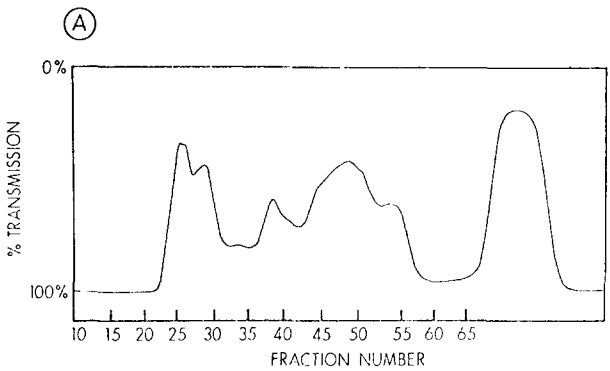
Dansylcadaverine incorporation into newborn skin in vitro

Analysis of the weights of the extracts (Table II) showed that protein was being converted to both the NaOH-soluble material and the final pellet. The amount of protein in the NaOH extract and pellet was lower in flask 2 than in flask 1 suggesting that dansylcadaverine may inhibit cross-linking into the cell pellet. Fluorescent patterns of the extracts after electrophoresis showed that dansylcadaverine had been incorporated predominantly into "high" and "very high" molecular weight material in the buffer, SDS and NaOH extracts. In addition, a 16 000 molecular weight protein in the buffer extract and a 52 000 molecular weight protein in the SDS extract incorporated dansylcadaverine. The intensity of the fluorescence of the 16 000 and 52 000 molecular weight proteins was much less than that of the higher molecular weight proteins (unpublished observations).

Incorporation of [^{14}C]putrescine into newborn rat epidermis in vitro

Epidermal proteins of newborn rat skin after an 18 h incubation with [^{14}C]-putrescine contained $1.5 \cdot 10^6$ acid-precipitable counts soluble in the buffer extract. After chromatography on 1.5 M agarose 400 000 cpm were recovered in the protein fractions. When the material from each of the peaks was electrophoresed on SDS-polyacrylamide gels and autoradiographed, the material from the first peak showed a large amount of [^{14}C]putrescine incorporation in the "very high" molecular weight material. The only other protein which incorporated [^{14}C]putrescine was in the fourth protein peak in a single radioactive band of molecular weight 13 000. The radioactivity in the band was much fainter in intensity than the high molecular weight bands, although this band corresponded to a region which stained heavily for protein. The material from the SDS extract was chromatographed on a 1.5 M agarose column and after autoradiography showed that [^{14}C]putrescine had been incorporated into only the very high molecular weight protein.

Fig. 1. SDS-polyacrylamide gel with extracts from crude homogenates of human scales incubated for varying lengths of time with 2 mM dansylcadaverine. The homogenates were extracted as outlined in Methods. Top (A) shows the gel stained for protein. Bottom (B) shows the fluorescent bands of the same gel. Slot No. 1 contains the molecular weight standards (S): bovine serum albumin (BSA), ovalbumin (OVA) and α-chymotrypsinogen (CHY). Slot Nos. 2—7 contain the buffer soluble proteins. Slot No. 2 is the control (C) (incubated 18 h with EDTA, iodoacetamide). Slot Nos. 3—7 were extracts of reaction mixtures incubated 35 min, 1, 2, 4 and 18 h, respectively. Slot Nos. 8—13 are the SDS extracts of the control (C) sample and the 35 min, 1, 2, 4, and 18 h reaction mixtures. Slot Nos. 14—19 are the NaOH extracts of the sample described above. Electrophoretic migration is from the top of the figure as indicated by the arrow. Fluorescent bands are the very high molecular weight proteins (VH), the high molecular weight proteins (H), and non-covalently linked dansylcadaverine at the solvent front (SF).



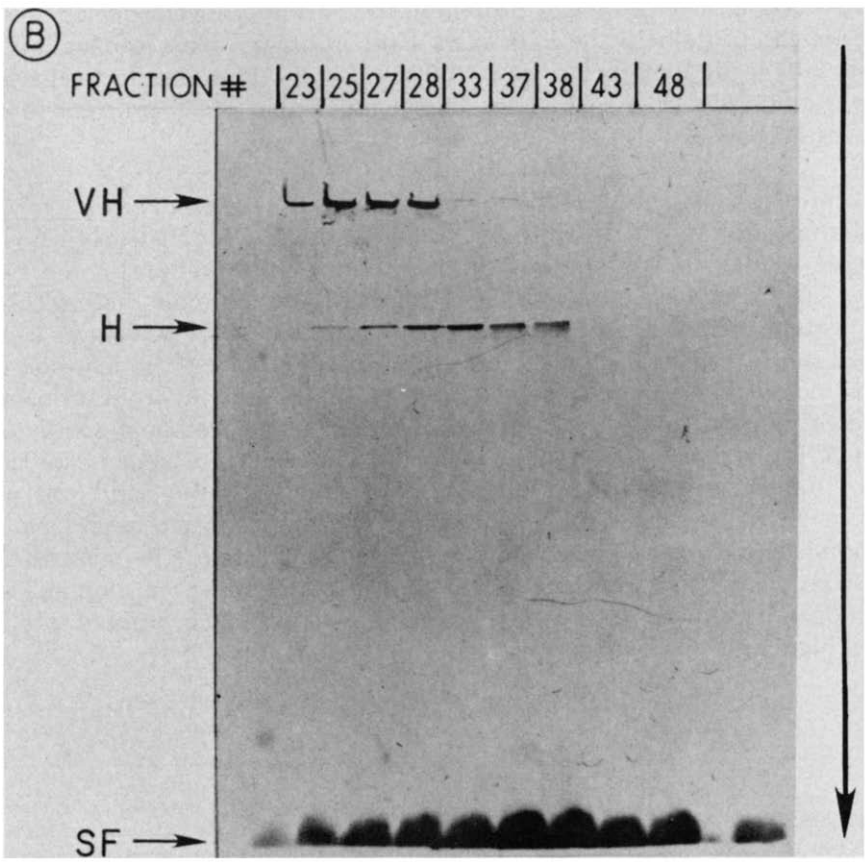


Fig. 2. Dansylcadaverine incorporation into human stratum corneum. Top (A) shows a direct tracing of the chromatography of an SDS extract of stratum corneum run on a 1.5 M agarose column as described in Methods. Bottom (B) is the fluorescent pattern of the column fractions when electrophoresed on 10% SDS-polyacrylamide gels. Electrophoretic migration is from the top of the figure as indicated by the arrow. VH indicates the very high molecular weight proteins, H high molecular weight proteins, and SF dansylcadaverine at the solvent front.

TABLE I

AMINO ACID ANALYSIS

Epidermis was extracted in neutral buffers or in neutral buffers containing SDS and dithiothreitol (SDS-DTT) as described in Methods. The proteins were fractioned on 1.5 M, 15 M or 150 M agarose as indicated in the legends. Protein refers to the gel electrophoresis pattern of the agarose-isolated proteins. High and very high are defined in results. The results are expressed in terms of residues per 1000 residues.

	Human epidermis		Rat epidermis					
	Extract							
	SDS-DTT	SDS-DTT	Buffer	SDS-DTT	SDS-DTT	SDS-DTT	SDS-DTT	NaOH
	Chromatography							
	1.5 M agarose	1.5 M agarose	1.5 M agarose	1.5 M agarose	1.5 M agarose	15 M agarose	150 M agarose	1.5 M agarose
	Protein							
	High and very high	High and very high	High and very high	High and very high	High	High and very high	Very high	Very high
Cysteic acid	8.9 *	34.4 *	14.5	9.6 **	15.8	16.9 **	33.5	44.3 *
Asx	81.2	85.9	91.3	66.4	65.2	70.8	65.4	69.1
Thr	16.6	34.1	44.5	48,6	57.2	40.7	37.8	42.1
Ser	121.4	120.6	83.7	136.4	144.0	125.7	127.0	123.0
Glx	155.2	150.8	198.3	180.2	150.6	163.3	142.2	140.0
Pro	34.1	31.3	42.8	29.2	24.9	26.4	39.4	32.0
Gly	224.6	192.2	110.6	164.6	145.6	185.5	203.6	172.3
Ala	52.2	45.2	75.3	72.5	81.4	63.1	67.9	64.8
Val	39.1	41.2	52.9	28.3	28.9	38.8	37.3	24.1
Met	6.7	—	—	2.8	3.0	—	—	—
Ile	34.1	34.7	36.2	23.6	28.5	33.3	24.8	26.4
Leu	70.1	74.7	60.9	43.7	45.7	53.9	50.7	54.0
Tyr	13.9	21.6	10.6	18.7	15.5	14.2	14.7	15.8
Phe	22.6	30.2	23.4	16.2	16.6	25.2	20.2	19.8
Lys	56.0	51.3	45.7	41.5	34.7	42.5	56.7	82.4
His	23.3	13.6	37.0	37.2	49.0	34.4	19.4	25.7
Arg	39.7	38.5	72.3	80.5	93.3	70.5	60.0	64.5

* Average of two preparations.

** Average of four preparations.

TABLE II

CROSS-LINKING OF NEWBORN RAT EPIDERMAL PROTEINS

The complete incubation and extraction conditions are in Methods.

Flask	Conditions	Weight (mg)			
		Buffer extract	SDS extract	NaOH extract	Final pellet
1	Calcium and dithiothreitol	19.4	160.5	27.5	26.6
2	Calcium, dithiothreitol and 2 mM dansylcadaverine	17.0	171.0	19.0	19.2
3	EDTA and iodoacetamide	45.6	175.0	6.2	16.0

Incorporation of ^{14}C-labelled amino acids in newborn rat epidermal protein and their in vitro cross-linking

^{14}C-labelled amino acids were incorporated into epidermal proteins which were in the buffer, SDS, NaOH extracts and in the final pellet. The specific activity (pmol/mg protein) of incorporated amino acids was highest for glycine. In the initial extracts (T_0) the specific activity for glycine was 2.7 and 0.4 for the NaOH extract and the final pellet, respectively. After 3 h incubation in the presence of calcium and dithiothreitol the specific activities in the same two extracts were 10.5 and 5.4; after 6 h, 11.3 and 4.2; and after 18 h, 10.3 and 10.0. The samples incubated in the presence of EDTA and iodoacetamide had no increase in the specific activity of glycine from 0 to 18 h. The buffer and SDS extracts had decreases in the specific activity of glycine from 3 to 18 h. The proline- and histidine-labelled proteins did not go through such dramatic changes in their specific activity during incubation.

Trypsin digestion of acceptor proteins

The [^{14}C]putrescine-labelled proteins from the buffer extract of newborn rat skin were digested with trypsin. During 1 h of trypsin incubation, the "very high" molecular weight proteins were digested to "high" molecular weight proteins. After an overnight incubation all of the "very high" molecular weight proteins had been digested. No other discrete radioactive bands were seen during the digestion. Protein stains showed the same pattern as did the autoradiographs.

In a similar experiment, the "very high" molecular weight proteins from an SDS extract of newborn rat epidermis labelled with dansylcadaverine were rapidly digested with trypsin. Within 1 min, all of the "high" and "very high" molecular weight material was completely digested, and all of the fluorescence moved with the dye front when electrophoresed. Within 3 min, all proteins larger than 25 000 daltons had been completely digested.

Amino acid analysis

Amino acid analysis of the "high" and "very high" molecular weight proteins from human stratum corneum showed that this material contained a large amount of glycine, glutamic acid, serine and aspartic acid (Table I). Glycine and glutamic acid alone accounted for 40% of the amino acid residues in the

protein. There was little variation in the amino acid composition, when the material was purified from an SDS-dithiothreitol extract of the scales was compared with material purified from a combined buffer and SDS-dithiothreitol extract of scales. The "high" and "very high" molecular weight proteins purified from the SDS-dithiothreitol extract were slightly enriched in cysteic acid threonine, serine and tyrosine, and they contained a slightly lower percentage of glycine and histidine.

Amino acid data from rat epidermis showed that the "high" and "very high" molecular weight material from all of the extracts contained large amounts of glycine, glutamic acid and serine, with glycine and glutamic acid alone comprising 30—34% of the amino acid residues. There were slight variations in the composition of the protein according to the solubility of the material and the size of agarose on which it was purified. Material from the buffer extract showed a high aspartic acid content when compared with the other samples. Protein which was extracted by SDS-dithiothreitol and purified on 1.5 M or 15 M agarose columns showed a slightly higher arginine content than all other samples. Analyzing the amino acid composition of samples in order of decreasing solubility (buffer extract, SDS-dithiothreitol extract purified on 1.5 M and 15 M agarose, SDS-dithiothreitol extract purified on 150 M agarose and NaOH extract) an increase was seen in the cysteic acid, glycine and lysine content, while there was a progressive decrease in the glutamic acid and possibly the histidine content of the protein.

Discussion

In these studies, potential substrates for epidermal transglutaminase have been isolated, and these proteins have been partially characterized in newborn rat and human epidermis. Two major proteins, or groups of proteins, incorporated [^{14}C]putrescine and dansylcadaverine; one group, designated "very high" molecular weight proteins did not enter 4% polyacrylamide gels; a second group, the "high" molecular weight proteins entered 4% polyacrylamide gels but not 10% polyacrylamide gels. These proteins were present in buffer, SDS and NaOH extracts. Occasionally, there were trace amounts of labelled lower molecular weight proteins. The incorporation of the labelled compounds was time dependent and inhibited by the known inhibitors of transglutaminases. On the basis of their solubility and molecular size the proteins are similar to the transglutaminase substrates in bovine epidermis [15].

During the incubation of newborn rat skin or stratum corneum some proteins had decreased solubility as shown by the increase in the amount of protein in the NaOH extract and the final pellet. Putrescine inhibited this cross-linking.

In an attempt to further characterize the specific proteins being cross-linked by transglutaminases, the changes in solubility of radiolabelled epidermal proteins was studied. Three amino acids were used: histidine, which is known to be incorporated into some of the keratohyalin proteins [16—18]; proline, which is found in high concentrations in the cell envelope [19]; and glycine, which is in high concentrations in several epidermal proteins. The major result was insolubilization of the glycine-labelled proteins; these results correspond with the

finding that amino acid compositions of the "high" and "very high" molecular weight proteins show them to have high glycine contents.

The "high" and "very high" molecular weight proteins were purified on SDS-agarose columns. The amino acid composition of the rat and human proteins from the buffer, SDS and NaOH extracts were similar suggesting they may all be related to each other. The differences in size and solubility may be related to the extent of ϵ-(γ-glutamyl)lysine cross-linking. The composition of these proteins does not match published analysis of any epidermal components or purified proteins. The amino acid composition of "high" and "very high" molecular weight proteins purified from human stratum corneum most closely resembles the analysis of human α-keratins [20], although α-keratins contain less glutamic acid and slightly more tyrosine than the proteins we have isolated.

The amino acid composition of the rat protein is similar to the composition of fibrous protein from newborn rat epidermis [21], although it is slightly higher in histidine, proline and arginine and has a somewhat lower leucine content. Our protein differs from the major protein of the cornified layer of newborn rat epidermis [22]. It is different from keratohyalin [23—26] or desmosomal proteins [27].

There is a possibility that the α-keratins are a major constituent of our "high" and "very high" molecular weight material based on the amino acid data. However, this hypothesis remains to be conclusively demonstrated by immunological identification and peptide mapping of the individual proteins.

The very high molecular weight protein was more susceptible to proteolysis than the high molecular weight protein. Proteins in the SDS extract were more sensitive than those in the buffer extract. The basis of the differing susceptibilities in the proteolysis is unknown. The relative resistance of the high molecular weight protein to proteolysis and its ability to incorporate putrescine make it a good candidate for a precursor of cell envelope [9]. The cell envelope is relatively resistant to proteolysis [28] and incorporates putrescine [9].

ϵ-(γ-Glutamyl)lysine peptides are in the urea-sulfhydryl-soluble protein of human epidermis which has an electrophoretic pattern similar to the material in the SDS-dithiothreitol extract from the present studies [10]. In light of the results from the present incorporation studies, it is possible the dipeptide is presently only in the "high" and "very high" molecular weight proteins in the urea-sulfhydryl extract.

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