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Authors

Thacher, Scott M
Rice, Robert H

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Keratinocyte-Specific Transglutaminase of Cultured Human Epidermal Cells: Relation to Cross-Linked Envelope Formation and Terminal Differentiation

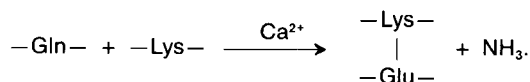
Scott M. Thacher* and Robert H. Rice
Charles A. Dana Laboratory of Toxicology
Harvard School of Public Health
665 Huntington Avenue
Boston, Massachusetts 02115

Summary

The predominant form of the cross-linking enzyme, transglutaminase, in cultured normal human epidermal keratinocytes, is found in cell particulate material and can be solubilized by nonionic detergent. It elutes as a single peak upon either anion-exchange or gel-filtration chromatography. Monoclonal antibodies raised to the particulate enzyme cross-react with one of two transglutaminases in the cell cytosol. The second cytosolic transglutaminase, which has distinct kinetic and physical properties from the first, does not cross-react and is not essential for formation of the keratinocyte cross-linked envelope *in vitro*. The anti-transglutaminase antibodies stain the more differentiated layers of epidermis in a pattern similar to that given by anti-involucrin antiserum. These observations support the hypothesis that the transglutaminase so identified is involved in cross-linked envelope formation *in vivo*.

Introduction

In cell culture, normal human epidermal keratinocytes form stratified colonies in which the differentiation program resembles that of epidermis *in vivo* (Green, 1980). During the final stage of differentiation, a layer of covalently cross-linked protein forms beneath the plasma membrane in the most superficial cells. The resistance of this cross-linked envelope to reducing agents and denaturants, such as SDS and urea, is due to extensive ϵ -(γ -glutamyl)-lysine isopeptide bonding (Rice and Green, 1977; Rothnagel and Rogers, 1984) formed by the enzyme transglutaminase, which links peptide-bound glutamine to primary amines such as the ϵ -NH₂ of lysine:



Envelope formation in cell culture can be induced by permeabilization of cells to calcium, a transglutaminase cofactor, and can be blocked by an excess of the transglutaminase substrate putrescine (Rice and Green, 1978). A precursor protein of the cultured keratinocyte envelope, named involucrin (Watt and Green, 1981), was initially identified as the primary cytosolic substrate for amine in-

corporation by transglutaminase and was subsequently shown to occur specifically in the most differentiated layers of epidermis (Rice and Green, 1979). Two other envelope precursor proteins, also keratinocyte-specific, are membrane-bound (Simon and Green, 1984).

Other transglutaminase-catalyzed reactions include stabilization of the fibrin clot (Folk and Finlayson, 1977; Aoki and Harpel, 1984; Lorand and Conrad, 1984), formation of the seminal plug in rodents (Williams-Ashman, 1984), ionophore-induced hardening of the erythrocyte membrane (Lorand et al., 1976), and possibly, cross-linking of lens proteins in cataracts (Lorand et al., 1981). The two best characterized enzymes, fibrin-stabilizing Factor XIII, which is synthesized as a zymogen, and the guinea pig liver or "tissue" transglutaminase, the function of which is unknown, differ both in structure and in reactivity toward certain glutamine-containing peptide substrates (Gorman and Folk, 1984). Both transglutaminases undergo a significant conformational change in the presence of millimolar calcium, which is necessary for full catalytic activity in all transglutaminases studied so far (Folk et al., 1967; Chung et al., 1974; Curtis et al., 1974; Fesus and Laki, 1977).

Initial experiments showed three chromatographically distinct transglutaminases in epidermal cell cultures, and we reasoned that these enzymes might exhibit kinetic, structural, and possibly functional differences. We observed that two of the transglutaminases were exclusively correlated with cross-linked envelope formation *in vitro*, and had substantially greater molecular weight than the 50–55 kd water-soluble enzymes that are extracted from human stratum corneum, bovine snout, and rat epidermis (Ogawa and Goldsmith, 1976 and 1977; Buxman and Wuepper, 1976; Peterson and Buxman, 1981). In characterizing the cultured keratinocyte enzymes, we identified a form that is expressed during human epidermal keratinocyte terminal differentiation.

Results

Characterization of Transglutaminase Activity from Cultured Human Keratinocytes

Homogenate fractions of human keratinocyte cultures, particulate as well as soluble, contain transglutaminase activity. In normal epidermal cells (strain LB) and in the squamous cell carcinoma line SCC-13, the majority of activity is located in cell particulates (Table 1). Approximately one-third of the activity was cytosolic in the LB cells, while ~5% was cytosolic in SCC-13. Enzymatic activity in 3T3 feeder cells was negligible in comparison to that in keratinocytes. Activity in the microsomal, (high-speed) pellet fractions was markedly less than in the particulate (low-speed) extracts.

The particulate activity of both the normal and the SCC-13 cells could be solubilized from the low-speed pellets by the nonionic detergent Emulgen 911, whereas little was extracted by homogenization or sonication in buffer with-

* Current address: National Institute of Dental Research, Building 30, Room 211, NIH, Bethesda, Maryland 20205.

Table 1. Distribution of Transglutaminase Activity in Extracts in Normal Epidermal and SCC-13 Keratinocytes

Fraction	Normal Epidermal (%)	SCC-13 (%)
Low speed pellet	29.5 (59)	42.5 (84)
Microsomal pellet	2.4 (5)	6.6 (13)
Supernatant	18.0 (36)	1.5 (3)

Confluent cultures were rinsed in 0.15 M NaCl-10 mM sodium phosphate, pH 7.2-1 mM EDTA, scraped into 2 mM EDTA-2 mM HEPES, pH 7.2 and disrupted by Dounce homogenization and sonication. A low speed pellet was recovered by centrifugation for 5 min at 2000 × g and rinsed in the EDTA-HEPES buffer. The combined cloudy supernatants were clarified at 100,000 × g for 1 hr to produce the microsomal pellet and the supernatant.

Units presented are nanomoles of putrescine incorporated/hr/confluent culture assayed in the absence of detergent (% of total activity in parentheses).

out detergent. To enumerate and characterize the transglutaminases present in these cells, cytosolic and particulate fractions were chromatographed on ion-exchange and gel-filtration columns with detergent present in all cases for purposes of comparison. As shown in Figure 1A, the cytosolic fraction of normal human epidermal keratinocytes has two forms of transglutaminase (Peaks I and II), which elute at 0.15 M and 0.3 M NaCl, respectively, from DEAE-cellulose anion-exchange columns. Transglutaminase extracted from normal cell particulates eluted at a position virtually identical with the supernatant Peak I (Figure 1B). The Peak I and particulate enzymes were, however, distinct molecules, as revealed by their behavior during gel filtration on Sepharose 4B. The particulate enzyme had an estimated Stokes radius of ~5.7 nm, and the soluble Peak I of ~4.3 nm, judging by the elution of catalase and lactate dehydrogenase employed as standards (Figure 2). The enzyme from SCC-13 particulate material was indistinguishable from the normal cell particulate enzyme in its elution from ion-exchange and gel-filtration columns, and preliminary analysis showed enzyme activity extracted from the "microsomal" fraction of SCC-13 cells behaved similarly.

In contrast to normal cells, Peak I, but not Peak II, activity was observed in SCC-13 cytosol, as shown by DEAE-cellulose chromatography (Figure 3). In addition, the Peak I enzyme of SCC-13 cells eluted at the same location as the Peak I enzyme of normal cells upon gel filtration. Thus both Peak I and particulate activities in the SCC-13 cell were comparable to their counterparts in cultured normal keratinocytes. The presence of Peak II transglutaminase in normal cells largely accounts for their greater total cytosolic activity compared with SCC-13 cells, as shown in Table 1.

Kinetic Comparison of Transglutaminases

The cytosolic Peak I enzyme of normal keratinocytes had a significantly higher K_M for methylated casein as substrate than the cytosolic Peak II enzyme (Figure 4). The K_M for the particulate enzyme, isolated by DEAE-cellulose chromatography, was 1.2 ± 0.2 mg/ml (3 determinations), close to that of the Peak I enzyme (1.5 ± 0.6 mg/ml, 3 determinations). Both differed from that of the

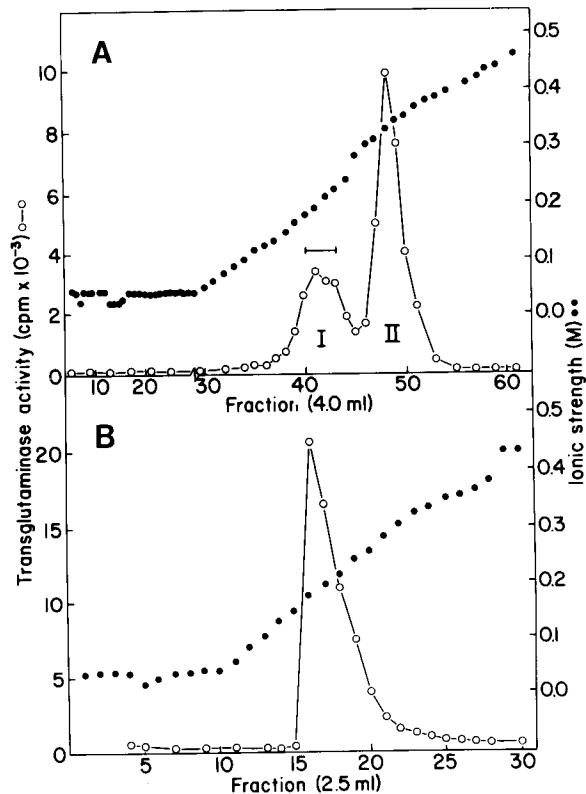


Figure 1. Cultured Human Epidermal Cell Transglutaminase: Anion-Exchange Separation

(A) The high-speed supernatant fraction derived from 20 confluent cultures of normal human keratinocytes was applied to a DEAE-cellulose column (15 ml volume) after addition of 0.1% Emulgen to the sample. The column was washed with 100 ml of equilibration buffer (50 mM Tris-Cl, pH 7.4-1 mM EDTA-1 mM DTE-0.1% Emulgen) and transglutaminase was eluted with a 0-0.5 M NaCl gradient (180 ml) in this buffer. Fractions 40-43 (indicated by the bar) were pooled and concentrated by ultrafiltration with an Amicon YM30 filter. (B) The particulate fraction of five confluent human keratinocyte cultures was extracted in 0.3% Emulgen, was chromatographed on DEAE-cellulose (5 ml volume), and was eluted with a 60 ml gradient as above.

Peak II enzyme (0.14 ± 0.05 mg/ml, 2 determinations) by an order of magnitude. In contrast, the Peak II and particulate enzymes had K_M values of 31 μ M and 36 μ M, respectively, for putrescine (at pH 8.3), and hence did not appear to be significantly different in this respect.

Reflecting its remarkably high content of Glx residues (Rice and Green, 1979), involucrin is an excellent substrate for the particulate enzyme of normal epidermal keratinocytes. In assays employing the DEAE-cellulose peak of particulate enzyme, [3 H]putrescine incorporation into involucrin was dependent upon the concentration of this substrate (Figure 5A) and was linear with time (Figure 5B). The results show a K_M for involucrin, the only radioactively labeled protein (Figure 5C), of <0.1 mg/ml, significantly less than that for methylated casein. In parallel assays with identical samples of transglutaminase, the relative V_{max} for putrescine incorporation into methylated casein was estimated to be 2 to 3 fold higher than for involucrin. However, the catalytic efficiency of the enzyme, defined as V_{max}/K_M (Folk and Finlayson, 1977), is con-

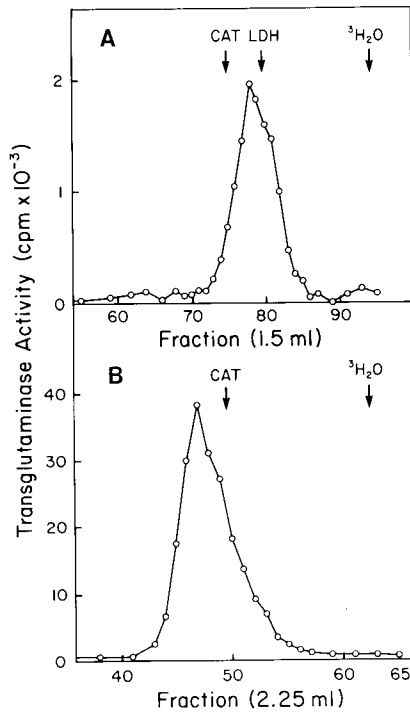


Figure 2. Cultured Human Epidermal Cell Transglutaminase: Gel Filtration

(A) Concentrated Peak I fractions (Figure 1A) were applied to a 1.6 × 70 cm column of Sepharose-4B maintained in 0.1 M NaCl–50 mM Tris-Cl, pH 7.4–1 mM EDTA–0.5 mM DTE–0.1% Emulgen buffer. (B) An Emulgen extract of particulate material from two keratinocyte cultures was run on the same column. The Emulgen extraction was carried out in the presence of 10 μg/ml each leupeptin and antipain. The positions of the markers bovine liver catalase (CAT), beef heart muscle lactate dehydrogenase (LDH), and ³H₂O are shown.

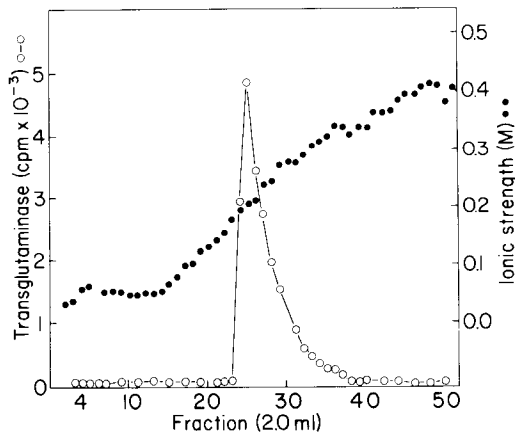


Figure 3. SCC-13 Cell Supernatant: Anion Exchange

The cytosolic extract of six SCC-13 cultures was chromatographed on DEAE-cellulose as in Figure 1A. Aliquots from the column were assayed in the presence of 10 μg/ml trypsin, omission of which did not qualitatively alter the profile.

siderably higher for involucrin than for methylated casein as substrate.

Because Peak II transglutaminase is absent from confluent SCC-13 cells, the majority of which are competent to form cross-linked envelopes when permeabilized to Ca²⁺ (Cline and Rice, 1983), this enzyme activity is not

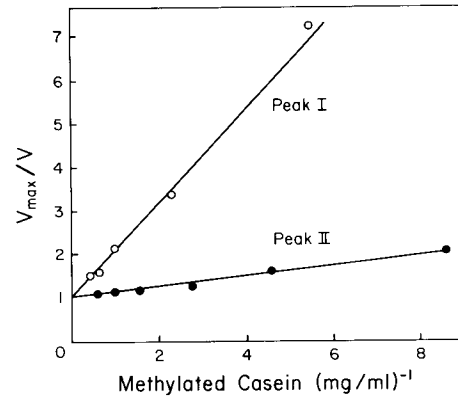


Figure 4. Lineweaver-Burke Analysis of Peak I and Peak II Transglutaminase for Methylated Casein Substrate

Peak I and Peak II enzymes obtained by DEAE-cellulose chromatography were pooled, concentrated, adjusted to pH 8.3, and assayed at the casein concentrations indicated. Each point is the median value of triplicate determinations, where V_{max} for each enzyme is normalized to 1.0 for comparative purposes. Standard assay conditions included 100 μM [³H]putrescine (6 μCi/ml), 0.04% Emulgen, 0.1 M NaCl, 2 mM DTE, 2 mM CaCl₂, 0.4 mM EDTA, 120 mM Tris-Cl, pH 8.3.

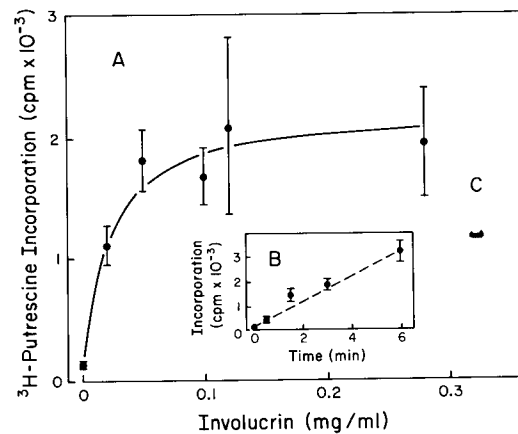


Figure 5. Involucrin as a Substrate for Particulate Transglutaminase

Detergent-extracted particulate transglutaminase of normal epidermal cells, partially purified on DEAE-cellulose, was incubated with involucrin of estimated 50% purity (following (NH₄)₂SO₄ precipitation—see Experimental Procedures) in siliconized tubes at 34.5°C with 40 μl final volumes containing 100 μM [³H]putrescine (1 μCi), 2 mM CaCl₂, 5 mM DTE, 120 mM Tris-Cl, pH 8.3. Reactions were stopped with 0.2 ml of 20 mM EDTA–2 mM putrescine–2 mg/ml casein and acidification with trichloroacetic acid. Values shown are the mean ± standard deviation of triplicate assays. Concentrations of involucrin were determined by Elisa modification (Parenteau and Rice, unpublished data) of a radioimmunoassay method (Cline and Rice, 1983).

(A) Dependence of acid precipitable radioactivity on involucrin concentration. (B) Time course of putrescine incorporation (0.1 mg/ml involucrin). (C) After 3 min incubation essentially as in (B), the reaction mixture was heated 1 min at 100°C with 20 μl of 20% SDS–60 mM EDTA and electrophoresed in 7% polyacrylamide gels. Shown here after autoradiography of the gel, virtually all the detectable radioactivity was seen in a band coinciding exactly with the stained band of involucrin (not shown), which had a mobility corresponding to approximately 130 kd. In parallel samples incubated either without involucrin or without transglutaminase, no radioactivity was detected.

quired for envelope formation *in vitro*. Normal cells trypsinized from the culture dish and washed extensively are also competent to form envelopes (Rice and Green, 1979), but we have found such cells lacked Peak II activity as de-

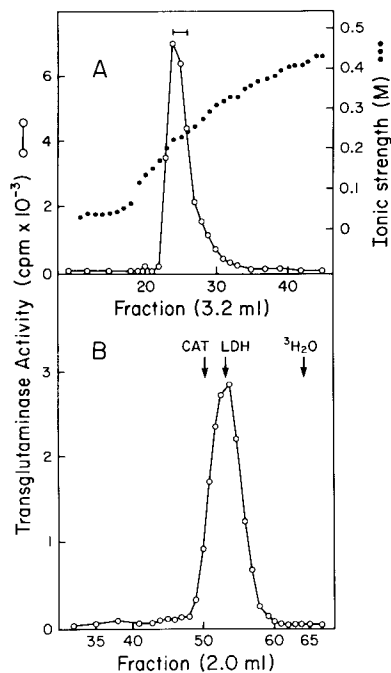


Figure 6. Transglutaminase of Trypsinized Human Epidermal Keratinocytes

Confluent cultures (6) were disaggregated by trypsinization (0.05% trypsin-0.25 mM EDTA in phosphate buffered saline, pH 7.1 [PBS] for 30 min at 37°C), diluted with an equal volume of medium supplemented with bovine serum (10%), recovered by centrifugation, and rinsed twice in medium containing serum and EDTA and once in PBS-1 mM EDTA.

(A) Dounce-homogenized cell cytosol was chromatographed on DEAE-cellulose in the presence of Emulgen as in Figure 1. (B) Peak I fractions (24-26) were pooled, concentrated, and applied to a Sepharose-4B column as in Figure 2, with the markers catalase (CAT), lactate dehydrogenase (LDH), and $^3\text{H}_2\text{O}$.

terminated by DEAE-cellulose chromatography (Figure 6A). Elution of the Peak I enzyme on gel filtration was essentially unaffected by the prior exposure of the cells to trypsin (Figure 6B), and the particulate transglutaminase was not destroyed by this treatment (data not shown).

Partial Purification of the Particulate Transglutaminase

Initial experiments showed that inclusion of 10 $\mu\text{g}/\text{ml}$ trypsin in assays of epidermal cell particulate transglutaminase, in the absence of detergent, stimulated activity 2 to 3 fold. Brief treatment of the particulate fraction of SCC-13 cells with 50 $\mu\text{g}/\text{ml}$ trypsin solubilized about 50% of the total activity, and a homogeneous peak of transglutaminase activity was obtained on Sepharose 4B at the approximate elution position of the Peak I soluble enzyme (Figure 7). Chromatography of the trypsinized enzyme on DEAE-cellulose was not observably changed. This form proved more stable than the detergent-extracted form during partial purification. A sequence of ammonium sulfate precipitation, anion exchange on DEAE-cellulose, and cation exchange on SP-Sephadex (Figure 8) resulted in a 30 to 40 fold increase in enzyme specific activity (Table 2). On several cation-exchange columns, such as SP-Sephadex,

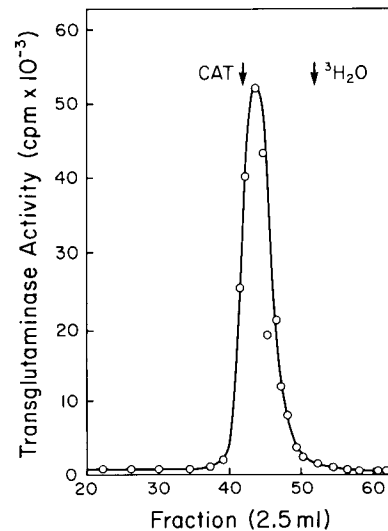


Figure 7. Gel Filtration of Trypsinized Particulate Transglutaminase
Particulate material from approximately 4 confluent SCC-13 cultures was trypsinized, clarified (100,000 \times g, 1 hr) and applied to a Sepharose 4B column (1.6 \times 52 cm) equilibrated with 50 mM Tris-Cl, pH 7.4-1 mM EDTA-0.1 M NaCl-1 mM DTE. Arrows show the elution positions of catalase (CAT) and $^3\text{H}_2\text{O}$. Approximately 100% recovery of activity was obtained.

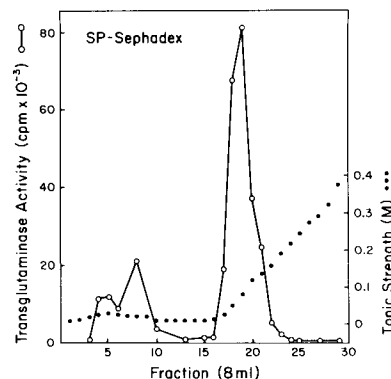


Figure 8. SP-Sephadex Chromatography of Partially Purified, Trypsinized SCC-13 Particulate Transglutaminase

Peak fractions from DEAE-cellulose were pooled and concentrated by ultrafiltration (Amicon YM30 filter) and the ionic strength was reduced by adding dilute buffer. The sample was then adjusted to pH 6.0 and applied to a column (1.4 \times 13 cm) of sulfopropyl Sephadex C-50 maintained in 25 mM 2-(N-morpholino)ethane sulfonate (MES), pH 6.0, 1 mM EDTA-0.5 mM DTE. After washing the column in this buffer, the sample was eluted with a linear gradient between the equilibration buffer and 0.2 M MES, pH 6.0-0.5 M NaCl-1 mM EDTA-0.5 mM DTE.

some heterogeneity was detectable as variable amounts of activity not bound to the resin, regardless of the initial trypsin treatment.

Immunochemical Characterization

The partially purified enzyme in the second peak from SP-Sephadex was used as an antigen for production of hybridoma cell lines. Spleen cells from immunized Balb/c mice were fused with NS-1 myeloma cells and screened for productive hybrids. Supernatants from positive cultures, in combination with rabbit anti-mouse IgG and inac-

Table 2. Partial Purification of Trypsin-Solubilized Particulate Transglutaminase

Fraction	Specific Activity (nmoles putrescine/ mg protein/hr)	Recovery (%)
Trypsin Extract	49	100
Ammonium Sulfate Precipitate	100	92
DEAE-Cellulose Column (pooled peak)	400	76
SP-Sephadex Column (pooled peak)	1800	10

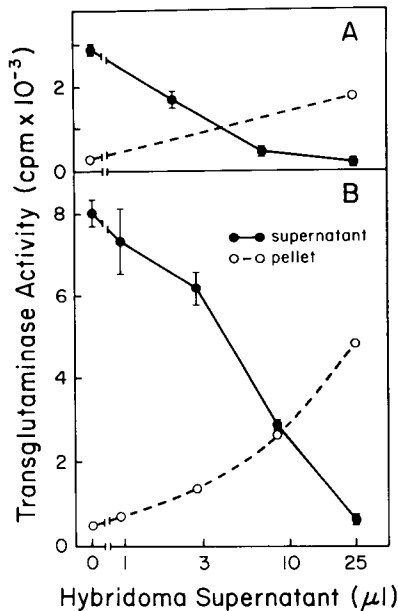


Figure 9. Immunoprecipitation of Normal Human Keratinocyte Transglutaminases

A complex of inactivated *S. aureus* (17 μl, 10% suspension), anti-mouse IgG (3 μl, 10 mg/ml total protein in IgG fraction), and monoclonal antibodies were incubated at 4°C, recovered by centrifugation, rinsed, and resuspended by sonication in Buffer C. Transglutaminase was added and after 30 min the complex was recovered by centrifugation. Both total supernatant (●) and antibody-precipitated (○) transglutaminase activities were assayed.

(A) Soluble Peak I transglutaminase isolated as in Figure 1A. (B) Detergent-extracted particulate transglutaminase of normal cultured human keratinocytes.

tivated *S. aureus*, precipitated detergent-solubilized transglutaminase. Figure 9 demonstrates the reaction of both particulate and Peak I supernatant transglutaminases from normal cells with one of three monoclonal antibodies obtained (B.D4).

Table 3 summarizes the specificity of three monoclonal antibodies raised against the SCC-13 particulate enzyme. Peak I cytosolic and particulate enzymes of the normal cells (and SCC-13 cells, as well) were all reactive with the antibodies, while the Peak II enzyme and transglutaminase from both soluble and particulate material of cultured human fibroblasts were not. One of the monoclonals, B.C1, immunoprecipitated activity from particulate extracts of cultured rat esophageal (Table 3) and endometrial cells (data not shown), which are highly competent to form

Table 3. Immunoreactivity of Transglutaminase Forms in Keratinocytes and Fibroblasts toward Three Monoclonal Antibodies

Transglutaminase Source	Activity Not Precipitated by Monoclonal Antibody (%)		
	B.D4	B.C1	I.B3
Human Epidermal Particulate	8.3	11.0	N.D. ^b
Cytosolic ^a Peak I	5.6	4.5	8.1
Peak II	80	84	86
Rat Esophageal Particulate	121	24	102
Human Dermal Fibroblast			
Cytosolic	-----98----- ^c		
Particulate	-----95----- ^c		

Extracts of the cells were fractionated into high speed (100,000 × g, 1 hr) supernatant (cytosolic) and pellet (particulate) fractions. Transglutaminase was solubilized from the particulate fractions with Emulgen 911 and, in parallel with cytosolic fractions, immunoprecipitated as described in Figure 9. The values presented give the % of activity not precipitated in comparison with nonimmune (NS-1 culture) supernatants employed in parallel as a control for each sample. Quantities of monoclonal antibody employed were in several-fold excess of that required to immunoprecipitate 50% of the particulate activity from normal cells. Recovery of 80–120% of the activity in the supernatant following immunoprecipitation was considered to show lack of reactivity.

^a From DEAE-cellulose chromatography.

^b Not determined in this experiment.

^c All 3 monoclonals were used simultaneously.

cross-linked envelopes (Heimann and Rice, 1983; Phillips and Rice, 1983).

These data suggest that the transglutaminase (or transglutaminases) so identified are specific to keratinocytes. Indirect immunofluorescent staining of skin by each of the three monoclonal antibodies was localized to the stratum corneum, and granular and upper spinous layers of the epidermis. Staining in the basal layer and dermis was at background levels. Figure 10 shows sections stained with the three monoclonals together, or normal mouse serum as a control, simultaneously with rabbit anti-involucrin antiserum, demonstrating transglutaminase localization with respect to a well studied marker of human keratinocyte differentiation (Rice and Green, 1979; Banks-Schlegel and Green, 1981; Rice and Thacher, 1985). Goat anti-rabbit IgG and goat anti-mouse IgG coupled to fluorescein and rhodamine, respectively, were used for counterstaining. Cross-reaction of the goat anti-rabbit IgG with the mouse monoclonal antibodies on these sections was negligible (not shown). The converse was not true, however, and it was necessary to pretreat the goat anti-mouse antibodies with normal rabbit serum to obtain a low background (Figure 10A). Figures 10B and 10C demonstrate the localization of transglutaminase with the monoclonal antibodies. In 10D, the same section as 10C is viewed with fluorescein, rather than rhodamine optics, to compare the distribution of involucrin and transglutaminase. The more basal extent of involucrin localization, as seen by comparison of Figures 10C, 10D, and other sections stained in the same way, suggests involucrin is expressed prior to transglutaminase by, at most, two cell layers during the keratinocyte differentiation program in skin. The greater sensitivity provided by the polyclonal anti-involucrin antiserum may, however, contribute to this disparity in localization of the two proteins.

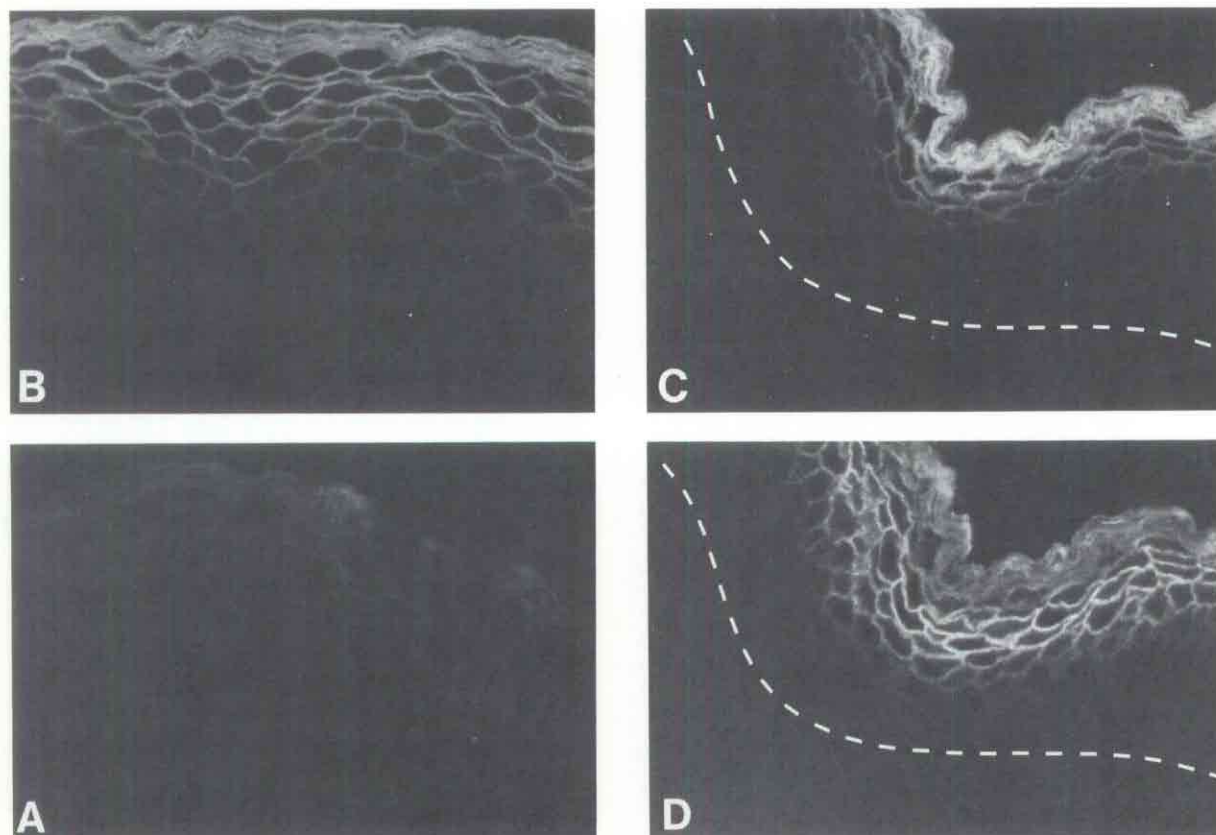


Figure 10. Immunofluorescent Localization of Transglutaminase and Involucrin

Frozen 4 μm sections of human foreskin were stained with the primary (1 $^\circ$) and secondary (2 $^\circ$) antibodies listed below. (A) 1 $^\circ$: rabbit anti-involucrin (1:400), nonimmune mouse serum (1:1000) in PBS; 2 $^\circ$: Rh $^+$ -GaM-IgG (goat anti-mouse IgG coupled to rhodamine), 1:400, rhodamine optics. (B) 1 $^\circ$: rabbit anti-involucrin (1:400) and I.B3 (1:1500 ascites), B.C1 (1:1500) ascites, B.D4 (1:2, hybridoma culture supernatant); 2 $^\circ$: Rh $^+$ -GaM-IgG, 1:400; goat anti-rabbit IgG coupled to fluorescein (1:1000), rhodamine optics. (C) As in (B). (D) The same section as (C), viewed by fluorescein rather than rhodamine optics.

To identify its molecular form, particulate transglutaminase was extracted from normal human epidermal keratinocytes metabolically labeled with [^{35}S]methionine. A single protein subunit of estimated 92 kd was specifically recovered in an immunoprecipitate in which the three monoclonals were used simultaneously (Figure 11, compare lanes 1 and 2); each monoclonal antibody individually gave the same result (Figure 11, lanes 3–7). The single radioactive protein that was specifically immunoprecipitated from the labeled cell cytosol (Figure 11, lanes 8 and 9) and corresponded to Peak I transglutaminase displayed the same apparent molecular weight as the particulate enzyme (Figure 11, lane 10).

The molecular weight of the SCC-13 cytosolic transglutaminase (Peak I) was estimated in the absence of denaturants or nonionic detergent. The enzyme had an estimated sedimentation coefficient ($S_{20,w}$) of 5.8 ± 0.2 S by sucrose density gradient analysis (Figure 12) and a Stokes radius of 4.55 ± 0.2 nm by gel filtration on Sepharose 4B with several markers in addition to catalase and lactate dehydrogenase. Its estimated native molecular weight calculated using the Svedberg equation (Martin and Ames, 1961; Siegel and Monty, 1966), assuming a partial specific volume of 0.735, is 115 ± 15 kd.

Discussion

We have identified a transglutaminase from cultured human epidermal cells that is present only in the more differentiated cells of epidermis and appears to be specific to the keratinocyte cell type. The immunoreactivity of particulate material from rat esophageal and endometrial cells with one of the monoclonal antibodies raised to the human enzyme indicates these cells express a similar transglutaminase. This finding suggests that the enzyme is a general component of cells with keratinocyte properties, and that it may be useful for studies of keratinocyte differentiation in nonprimate animals such as the rat, where it has not been possible to find an involucrin-like protein (Rice and Thacher, 1985).

In cell culture, the transglutaminase occurs in two forms: one, comprising the majority of activity, appears to be membrane-bound; the other is cytosolic. Both contain 92 kd protein subunits of apparently identical mobility on SDS polyacrylamide gels (Figure 11). These two enzymes must differ at the molecular level since the particulate form, solubilized by nonionic detergent, has a larger Stokes radius than the cytosolic, as determined by gel filtration in the presence of detergent. A detergent-binding

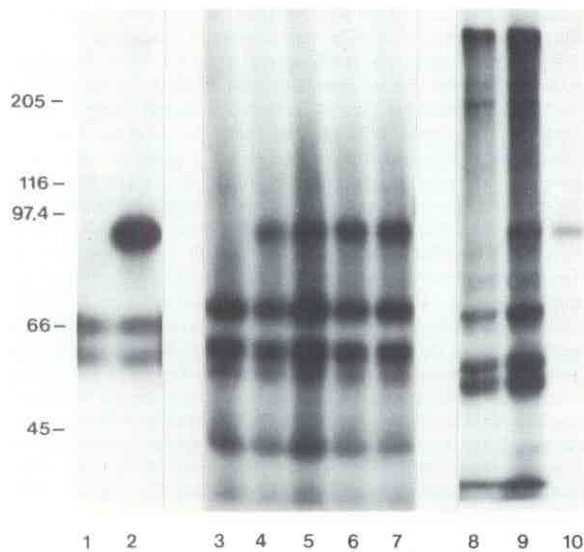


Figure 11. Immunoprecipitation of [³⁵S]Methionine Labeled Transglutaminase

Preconfluent normal human keratinocytes or SCC-13 cells, in individual wells of a 24-well plate, received approximately 0.4 ml of methionine-free medium (Gibco, Selectamine Kit) supplemented with fetal bovine serum (3%), hydrocortisone (0.4 μg/ml), and 50–100 μCi of [³⁵S]Methionine (New England Nuclear, Boston, MA) for periods of 4–12 hr, followed by a brief chase (30 min–4 hr) with normal cell growth medium. Cells were scraped from the dish in 2 mM EDTA–2 mM HEPES, pH 7.2 with 10 μg/ml each leupeptin and antipain and frozen at –70°C. Freeze-thawed (3x) cells were dispersed with a bath sonicator and centrifuged at 25,000 × g for 20 min to separate cytosol and particulate fractions. The particulate fraction was sonicated in the presence of 0.3% Emulgen and cleared as above. Immunoprecipitates of either *S. aureus*, rabbit anti-mouse IgG, and monoclonal antibody (lanes 1–7) or rabbit anti-mouse IgG immunobeads (BioRad) and monoclonal antibody (lanes 8–10) were washed twice in 0.8 ml of 20 mM Tris–Cl, pH 8.0–1 mM EDTA–0.1% Emulgen, twice in 0.8 ml of 50 mM Tris–Cl, pH 8.0–1 mM EDTA–1% Emulgen–0.5 M NaCl, and once in 0.1 ml of distilled water. The pellet was then dispersed in buffer containing SDS, boiled, and electrophoresed on 7.5% SDS polyacrylamide gels.

Immunoprecipitates were of cell particulate detergent extracts (lanes 1–7 and 10) or cell cytosol (lanes 8 and 9). Lanes 1, 3, and 8 were controls with no monoclonal antibody; lanes 2, 7, 9, and 10 used the combined monoclonal antibodies I.B3, B.C1, and B.D4; lane 4, I.B3 alone; lane 5, B.C1 alone; lane 6, B.D4 alone.

The following molecular weight standards (Sigma) on the stained gels were used for molecular weight estimation: myosin (200 kd), β-galactosidase (116 kd), phosphorylase a (97.4 kd), bovine serum albumin (66 kd), and ovalbumin (45 kd).

site on the particulate form, such as covalently-bound fatty acid (Kaufman et al., 1984), could account for the difference and for the particulate localization of the enzyme, although it cannot be discounted that the two transglutaminases are separately coded polypeptides. Trypsinization (and solubilization) of the particulate transglutaminase produces a molecule similar to cytosolic Peak I in gel-filtration properties. It is possible that proteolytic cleavage removes a small peptide containing a membrane- or detergent-binding site at one end of the protein, so that it is roughly equivalent to the Peak I enzyme. Physical study of the cytosolic enzyme indicates, well within the margin of error, that it contains a single 92 kd subunit.

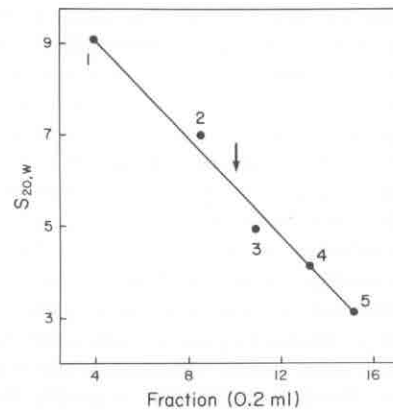


Figure 12. Sucrose Density Gradient Analysis of Supernatant Transglutaminase

The $S_{20,w}$ values of marker proteins: 1, fumarase; 2, keratinocyte lactate dehydrogenase; 3, transferrin; 4, human hemoglobin; and 5, carbonic anhydrase are plotted against the fraction number of peak activity. The location of maximal transglutaminase activity is indicated by the arrow. Sucrose gradients (5–20%) in 50 mM Tris–Cl pH 7.4–1.0 mM MgCl₂–0.4 mM EGTA–2 mM DTE were centrifuged at 48,000 rpm for 11.5 hr in a SW 50.1 swinging bucket rotor.

The existence of particulate transglutaminase activity has been reported in a number of tissues (Birckbichler et al., 1977; Griffin et al., 1978; Folk, 1980), including chick epidermis (Bures and Goldsmith, 1978), but neither the structural nature nor physiological importance have been examined. Since the particulate transglutaminase of human fibroblasts is immunologically distinct from the activity described here (Table 3), it is possible that there are several types of particulate transglutaminases; conceivably, a close relation between soluble and particulate forms may occur in other systems as well.

We have been unable to relate the 92 kd enzyme to a 51 kd transglutaminase reported to be a component of human stratum corneum (Ogawa and Goldsmith, 1976). None of the positive hybridoma supernatants that we obtained immunoprecipitated transglutaminase activity in aqueous extracts of human stratum corneum. Immunofluorescent localization of the 51 kd enzyme in human epidermis has not been reported, and therefore the 92 kd enzyme described here cannot be compared to it on that basis. However, there is some evidence to suggest that the 55 kd transglutaminases from bovine snout and newborn rat epidermis are localized in the more peripheral layers of these epithelia (Buxman and Wuepper, 1975 and 1978; Peterson and Buxman, 1981). It is possible that breakdown of the 92 kd enzyme produces the 51 kd form, but that none of the epitopes to which the monoclonal antibodies are directed is retained. There is good evidence for partial cleavage of keratins in the stratum corneum (Bowden and Cunliffe, 1981), which may be due, in part, to the release of hydrolytic enzymes by the terminally differentiating keratinocyte (Lavker and Matoltsy, 1970).

One form of transglutaminase in the cytosol of normal human keratinocytes, Peak II, is not present in SCC-13 cells or trypsinized cultured human epidermal cells, both of which can be stimulated to form cross-linked envelopes by calcium permeabilization. The Peak II enzyme is there-

fore not essential for cross-linked envelope formation *in vitro*. Since Peak II has a K_M for casein different from that of Peak I and particulate enzymes, it may have other functions in the cell, but its participation in the formation of a properly resilient squame *in vivo* is not ruled out.

A limited survey of several keratinocyte lines derived from human squamous carcinomas (Rheinwald and Beckett, 1981) has shown that the particulate/Peak I and the Peak II activities can be regulated quite differently. In SCC-13, which does not express Peak II transglutaminase, the enzyme activity is suppressed by retinoids (Thacher, Coe, and Rice, 1985) and by calcium deprivation; these responses also occur in SCC-12B, but the Peak II enzyme, which is expressed in this cell line, is greatly stimulated by retinoids (Rubin and Rice, unpublished data). The phenomenon of Ca^{2+} and retinoic acid stimulation of transglutaminase activity was initially demonstrated in mouse epidermal primary cell culture (Yuspa et al., 1981; Yuspa et al., 1982). Two separate transglutaminase forms, corresponding in elution from cation-exchange columns to the Peak I/particulate and Peak II transglutaminases, have also been described in the mouse cells, where they are stimulated by Ca^{2+} and retinoic acid, respectively (Lichti et al., 1985). Retinoic acid stimulation of transglutaminase is accompanied by a decrease in spontaneous envelope formation (Yuspa et al., 1982), illustrating that expression of Peak II enzyme activity is not linked to envelope formation. This may simply reflect the fact that the Peak II enzyme can be expressed in an epidermal keratinocyte in the absence of necessary envelope precursor proteins. It is likely that the Peak II enzyme corresponds to the guinea pig liver or "tissue" type of transglutaminase, which has been found in human blood cells (Brenner and Wold, 1978; Murtaugh et al., 1984) and a wide variety of guinea pig tissues (Chung, 1972). It requires a similarly high ionic strength for elution from DEAE-cellulose (Tyler and Laki, 1966), and is substantially increased in mouse macrophages by exposure to retinoic acid (Moore et al., 1984). The disappearance of this enzyme upon incubation of cultured cells in trypsin merits further study.

In the SCC-13 cell, the Peak I and particulate enzymes are regulated roughly in parallel to each other and to envelope competence (the percent of cells capable of forming envelopes upon Ca^{2+} -ionophore stimulation) in response to the opposing effects in culture of hydrocortisone and retinoids (Thacher, Coe, and Rice, 1985). However, we do not know the relative importance of the two enzymes in envelope formation, nor can we say what form the enzyme takes in epidermis. Since some precursor proteins of the cross-linked envelope are membrane-bound (Simon and Green, 1984), our results suggest that particulate transglutaminase, which is, by far, the most active of the two, is the most favorably positioned to cross-link them. This interpretation appears fully compatible with the enzymatic cross-linking of proteins by keratinocyte particulates *in vitro* (Simon and Green, 1985).

Experimental Procedures

Cell Culture

Keratinocytes (strain LB) from normal human epidermis were cultivated in 10 cm plastic dishes with 3T3 feeder layer support according

to standard methods (Rheinwald, 1980) in Dulbecco-Vogt Eagle's and Ham's F12 media (a 3:1 mixture) supplemented with fetal bovine serum (5%), hydrocortisone (0.4 μ g/ml), epidermal growth factor (10 ng/ml), adenine (0.18 mM), triiodothyronine (20 pM), insulin (5 μ g/ml), transferrin (5 μ g/ml), penicillin (0.1 mg/ml), and streptomycin (0.1 mg/ml). Squamous carcinoma cells (SCC-13) were cultivated in the same fashion (Rheinwald and Beckett, 1981) in Dulbecco-Vogt Eagle's medium supplemented with fetal bovine serum (1%) and hydrocortisone (0.4 μ g/ml), which is required for full transglutaminase expression (Thacher, Coe, and Rice, 1985). SCC-13 was also cultivated, on a larger scale, in plastic or glass roller bottles, the latter pretreated for 1 day with bovine serum (12 ml each). The cells were inoculated in medium supplemented with fetal bovine (1%) and bovine (10%) sera and maintained in medium containing fetal bovine (1%) and bovine (5%) sera and hydrocortisone (0.4 μ g/ml). The bovine serum supplement, while unnecessary for growth in plastic dishes, assisted attachment and prevented sloughing of confluent cultures from the roller bottles. Human dermal fibroblasts and 3T3-J2 cells were grown in medium supplemented with bovine serum (10%). Rat esophageal and endometrial epithelial cells were those previously described (Heimann and Rice, 1983; Phillips and Rice, 1983) and cultivated as the normal human epidermal cells with addition of cholera toxin (9 ng/ml) to the medium.

Purification of Involucrin

Confluent normal epidermal cultures (stored frozen) were disrupted by Dounce homogenization and centrifuged at $100,000 \times g$ for 1 hr. The supernatant was dialyzed against 3 changes of 1 mM EDTA-1 mM citrate buffer, pH 6.0, adjusted to pH 5.1, clarified ($10,000 \times g$, 15 min), and adjusted to pH 7.0 with subsequent addition of $(NH_4)_2SO_4$ (0.24 g/ml). The precipitate, containing involucrin of estimated 50% purity by gel electrophoresis, was recovered by centrifugation ($10,000 \times g$, 15 min). Such preparations were either dialyzed extensively against 5 mM Tris buffer, pH 7.4-1 mM EDTA and stored frozen or purified to homogeneity by gel-filtration and DEAE-cellulose chromatography (Rice and Green, 1979). All fractionation steps were performed at 4°C. Purified involucrin was used to elicit a specific rabbit antiserum, which reacts only with involucrin in extracts of human epidermis examined by immunoblotting of SDS gels (Rice and Thacher, 1985).

Solubilization of Particulate Transglutaminase

Transglutaminase was solubilized from the particulate fraction (Table 1) of SCC-13 or normal human epidermal cells by resuspending and incubating high speed pellets ($100,000 \times g$, 1 hr) at 0°C for 30 min in 2 mM HEPES, pH 7.2-2 mM EDTA-2 mM DTE-0.3% Emulgen 911 (generously provided by John Dent, CIIT, Research Triangle Park, NC), 1.5 ml/culture. Alternately, SCC-13 particulate material was resuspended in 50 mM Tris-Cl, pH 7.4-1 mM EDTA-1 mM DTE, 0.6 ml/culture or equivalent. The latter samples were treated for 90 sec at room temperature with 50 μ g/ml TPCK-trypsin (Worthington Biochem. Corp., Freehold, NJ), after which the digestion was stopped with soybean trypsin inhibitor (0.15 mg/ml). Shorter trypsin incubations appeared adequate to solubilize transglutaminase, but a lower concentration of trypsin (10 μ g/ml) was not reproducibly as effective. After either treatment, the sample was then clarified ($100,000 \times g$, 1 hr).

Partial Purification of Transglutaminase on DEAE-Cellulose

The following three-step procedure, carried out at 4°C was employed to purify the trypsin-solubilized enzyme from the equivalent of 200 SCC-13 cultures. A precipitate following addition of $(NH_4)_2SO_4$ (0.15 g/ml) was discarded, and a second precipitate was recovered by centrifugation ($10,000 \times g$, 10 min) after further addition of $(NH_4)_2SO_4$ (0.25 g/ml initial volume). This material was solubilized in 50 ml of 50 mM Tris-Cl, pH 7.4-1 mM EDTA-2 mM DTE-5 μ g/ml each leupeptin and antipain, and dialyzed for several hours against 5 mM Tris-Cl, pH 7.4-2 mM EDTA-5 mM β -mercaptoethanol. A DE-52 (Whatman) column (2.5 cm \times 5 cm) was equilibrated with 50 mM Tris, pH 7.4-1 mM EDTA-0.3 mM DTE. After application of the dialyzed sample, the column was rinsed with 100 ml of equilibration buffer and eluted with a 250 ml gradient of 0-0.5 M NaCl in 50 mM Tris-1 mM EDTA-0.5 mM DTE. Peak fractions were pooled and purified further on SP-Sephadex (Figure 6).

Transglutaminase Assay

Transglutaminase was assayed by incorporation of [3H]putrescine (8 μ M, 250 Ci/mol) into 2 mg/ml reductively methylated (Means and

Feeney, 1968; Lorand et al., 1974) α -casein (Worthington) in 100 mM Tris-Cl, pH 8.3–2 mM CaCl_2 –2 mM DTE–Emulgen 911 (0–0.1%). Aliquots of column fractions (0.1–0.2 ml) or other samples were incubated 10–30 min in final volumes of 0.25 ml at 35°C (in the linear range of the assay). After acidification with trichloroacetic acid (10%), the precipitated casein was recovered on Whatman GF/A filters, rinsed, and scintillation counted (Thacher, Coe, and Rice, 1985). To calculate kinetic parameters, absolute casein concentrations were determined by treatment with ninhydrin after acid hydrolysis (Schiffman, 1966).

Preparation of Hybridomas

Female Balb/c mice were injected with partially purified transglutaminase (80 μg of protein) in suspension with Freund's complete adjuvant and 80 μg of Poly(I)-Poly(C) ribonucleic acid (Sigma). An additional injection of 80 μg of protein was made 25 days later in Freund's incomplete adjuvant. Final injections of protein in PBS were made both intraperitoneally and intravenously 3½–4 days prior to sacrifice. Spleen cells were fused with P3/NS1/1-AG4-1 (NS-1) myeloma cells by standard methods (Kohler and Milstein, 1976; Lake et al., 1979; Burden, 1982), distributed in four 24-well plates containing at least 10^4 mouse macrophages per well as feeder cells, and grown in a hypoxanthine-aminopterin-thymidine selective medium (Burden, 1982). Ten to fourteen days later, cell supernatants were screened by placing a 30 μl drop on small squares of nitrocellulose paper spotted with 0.3–1.0 μg of the injected antigen, and were blotted with 5% fetal bovine serum in PBS to prevent nonspecific protein absorption. Adhering mouse antibody was detected by reaction with rabbit anti-mouse IgG coupled to peroxidase (Towbin et al., 1979; Simon and Green, 1984). Wells positive for the antigen were assayed further by immunoprecipitation of detergent-extracted SCC-13 transglutaminase activity (see below). Hybridoma wells found to be positive in this assay were subcloned at least twice. To elicit ascites fluid, approximately 0.5 – 2.0×10^6 cells were injected intraperitoneally into mice primed 3–7 days earlier with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane). One of the monoclonals, B.C1, is an IgG_{2a}; B.D4 and I.B3 are of the IgG₁ type, as determined with type-specific rabbit antibodies (Miles) and goat anti-rabbit IgG coupled to peroxidase (BioRad) in the nitrocellulose blotting assay.

Immunoprecipitation of Transglutaminase

Transglutaminase samples (about 4000 cpm in a 30 min assay) were incubated with monoclonal antibody from hybridoma culture supernatant or diluted ascites fluid at 4°C for 30 min in the presence of leupeptin and antipain (10 $\mu\text{g}/\text{ml}$ each)–0.3% Emulgen–20 mM Tris-Cl, pH 7.4–0.2 M NaCl (Buffer C) in capped 1.5 ml polypropylene tubes. Addition of rabbit anti-mouse IgG (IgG fraction, Cappel Laboratories) was followed 15 min later by formalin-treated and heat-inactivated *Staph aureus* (IgSorb, The Enzyme Center, Malden, MA) and the complex was recovered by centrifugation after 15 min. The pellet was rinsed gently with Buffer C to remove traces of the supernatant and was dispersed in a sonicating water bath in the presence of transglutaminase assay components to determine the extent of enzyme immunoprecipitation. NS-1 culture supernatant or 15% fetal bovine serum replaced the monoclonal antibodies in control immunoprecipitations. This method, a variation of which is presented in Figure 9, permitted rapid initial screening of hybridomas.

Immunofluorescent Staining

Circumcised human foreskin was trimmed of fat, coated in O.C.T. embedding medium, and dropped into isopentane cooled in liquid N₂. Sections of 4 μm were cut at –20°C, placed on glass slides (previously dipped in 0.5% gelatin–0.05% chrome alum at 56°C and dried), and used shortly thereafter or stored at –70°C with dessicant. Slides were incubated in Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.4–1 mM EDTA–0.2 M NaCl–0.02% NaN₃) for 15 min, incubated with 80 μl of various antibody preparations for 2 hr at room temperature, washed in TBS (3 \times , 5 min), and incubated 1 hr with each of two chromophore-coupled indicator antibodies (with three 5-min washes in between): rhodamine-conjugated IgG fraction of goat anti-mouse IgG antiserum and fluorescein-conjugated IgG fraction of goat anti-rabbit IgG antiserum (Cappel Laboratories) at dilutions of 1:400 and 1:1000, respectively. The anti-mouse IgG antibodies had detectable anti-rabbit IgG immunoreactivity, which was neutralized by preincubating a 1:200 dilution of the conjugated antibody with a 1:200 dilution of rabbit serum.

Fluorescent sections were photographed on a Zeiss photomicroscope (Burden, 1982).

Determination of Physical Parameters

The following $s_{20,w}$ and diffusion ($d_{20,w}$ in units of 10^{-7} cm²/sec) coefficients (Smith, 1970; Brewer et al., 1974) were employed for protein markers in sucrose density gradient (Martin and Ames, 1961) and gel-filtration (Siegel and Monty, 1966) analysis; *E. coli* β -galactosidase (Sigma) $D = 3.12$; porcine heart fumarase (Sigma) 9.09 S, $D = 4.05$; bovine liver catalase (Sigma) $D = 4.1$; yeast alcohol dehydrogenase (Sigma) $D = 5.13$; bovine heart lactate dehydrogenase (Sigma) 7.0 S, $D = 5.1$ (values for the human keratinocyte enzyme are assumed to be identical with those of bovine heart); human transferrin (Sigma) 4.92 S, $D = 5.85$; human hemoglobin (Calbiochem) 4.13 S; bovine red blood cell carbonic anhydrase (Sigma) 2.9 S, $D = 9.0$; horse heart cytochrome c (Sigma) $D = 12.3$. Stokes radii, inversely proportional to diffusion coefficients, were calculated as described (Siegel and Monty, 1966). In gradient and column runs, markers were located according to standard assay procedures (Worthington Enzyme Manual, Freehold, NJ), by SDS gel electrophoresis, or in the case of fumarase by the method of Aiyer (1983).

SDS Gel Electrophoresis and Fluorography

Electrophoresis in polyacrylamide slab gels in the presence of SDS was performed as described by Laemmli (1970). The gels were stained with Coomassie Blue, impregnated with Enhance (New England Nuclear, Boston, MA), dried, and exposed to Kodak XAR-5 film for several days.

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