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High-Molecular-Weight Human Epidermal Transglutaminase*

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Human stratum corneum was extracted in Tris-HCl containing EDTA and phenylmethylsulfonyl fluoride, separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transblotted to nitrocellulose papers and reacted with rabbit antihuman epidermal transglutaminase (ETG) antibody. Protein-bound antibody was detected with a multistep peroxidase procedure. Proteins with a molecular weight of 50,000 (50kDa) and 72,000 daltons (72kDa) were stained when anti-ETG was used and not when second antibody alone or sera from nonimmunized animals were used. When ETG was treated with trypsin or organic solvents, there was no alteration in the mobility of the 50kDa ETG band, but there was complete disappearance of the 72kDa band. Antibody that bound 72kDa protein, when eluted from the blot, reacted with both 50kDa and 72kDa proteins; similarly, antibody that bound to the 50kDa protein, when eluted from the blot, reacted with both the 50kDa and 72kDa proteins. Partially purified 72kDa ETG activity was increased (3 to 16 times control levels) after heating at 56°C in the presence of calcium and dithiothreitol or by treatment with trypsin.

These studies, in conjunction with the previous studies of ETG activation, are consistent with there being two forms of ETG. The different forms may play a role in regulating enzyme activity.

The transglutaminases (R-glutaminyl-peptide:–amine–γ-glutamyl transferase, E.C. 2.3.2.13), a heterogenous group of Ca^{++} dependent enzymes, catalyze the formation of γ-glutamyl-ϵ-lysine isopeptide bonds which can cross-link proteins. These enzymes are biochemically and immunochemically distinct [1,2], and multiple forms of transglutaminase can exist within the same tissue [3].

Regulation of epidermal transglutaminase (ETG) determines the timing and extent of cell envelope formation (reviewed in [4]). Activation [4–7] or inhibition [4] of transglutaminase activity may directly affect cell envelope formation in keratinocytes in tissue culture. Some treatments can increase transglutaminase activity and not increase cornified envelope formation [8].

In this paper we describe a new high-molecular-weight form of human ETG.

MATERIALS AND METHODS

[1,4-^{14}C]Putrescine dihydrochloride (109 mCi/mmol) was purchased from Amersham (Arlington Heights, Illinois) and Aquasol-II from New England Nuclear (Boston, Massachusetts). α-Casein, *Staphylococcus aureus* V8 protease, and trypsin-TPCK were obtained from Worthington Biochemical Co. (Freehold, New Jersey). Phenylmethylsulfonyl fluoride (PMSF), plasmin, ethylenediaminetetraacetic acid (EDTA), chymotrypsin, 4-chloro-1-naphthol, were from Sigma Chemical Company, (St. Louis, Missouri). Plasminogen activator (Winkinase, 250,000 CTA units) was kindly provided by Dr. Gerald Lazarus, Department of Dermatology, University of Pennsylvania. Peroxidase-conjugated affinity-purified goat antirabbit IgG was purchased from Cappel Laboratories (Cochranville, Pennsyvania). All other reagents were of the highest grades available.

Polyclonal rabbit antibody for human ETG was prepared by Ogawa and Goldsmith [2].

Transglutaminase Preparation

Transglutaminase was prepared from human skin scales obtained from a patient with lamellar ichthyosis. Scales were finely minced with scissors, homogenized in 10 vol of 0.25 M sucrose, and centrifuged at 17,000 *g* for 15 min at 4°C. The supernatant solution was saved and the pellet rehomogenized in neutral buffer containing 0.1 M Tris-HCl, pH 8.0, 0.15 M NaCl, with or without 1 mM EDTA and 0.1mM PMSF, and centrifuged as above. Both supernatants were combined and concentrated 10-fold by ultrafiltration using an Amicon YM-5 filter at 4°C.

Neonatal foreskin epidermis was prepared by heating to 56°C for 30 s in distilled water and mechanically separating epidermis from dermis. The epidermis was extracted in 0.1 M Tris-HCl, pH 8.0, with 0.15 M NaCl, 1 mM EDTA, and 0.1 mM PMSF. Foreskin epidermal cells were grown in tissue culture and the keratinocytes extracted directly into the same buffer, or in some cases extracted as foreskin.

Protein Separation and Immunoperoxidase Staining

Extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [9] and gels were either stained or transblotted to sheets of nitrocellulose [10]. Molecular weight standards were bovine serum albumin, egg albumin, carbonic anhydrase, and trypsin inhibitor. Gel blots were processed for immunologic studies and reacted with rabbit antihuman transglutaminase [2] followed by goat antirabbit IgG horseradish peroxidase-conjugated second antibody. Horseradish peroxidase was developed using 4-chloro-1-naphthol in 0.01% H_2O_2 [11].

Cross-Reactivity of Anti-50kDa and Anti-72kDa Transglutaminase Antibody

Specific antibodies, anti-50kDa and anti-72kDa transglutaminase antibody, were prepared from whole rabbit antitransglutaminase antisera using affinity purification as described by Olmsted [12]. Briefly, extracted proteins were separated on SDS-PAGE, transferred to a nitrocellulose sheet, and reacted with antitransglutaminase antibody at room temperature overnight. After locating transglutaminase by immunoperoxidase staining on a portion of the blot, the 50kDa, 72kDa, and control zones were cut out from the nonstained remainder of the blot. These strips were then washed with several changes of elution buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.15 M NaCl) and placed in separate 5-cc syringes. Three milliliters of 0.2 M glycine-HCl, ph 2.8, was added to the syringe and left for 3 min. This solution was forced

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Abbreviations:
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
ETG: epidermal transglutaminase
HPLC: high-pressure liquid chromatography
kDa: kilodaltons
PMSF: phenylmethylsulfonyl fluoride
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

through the strips twice and then neutralized with 1 N NaOH. The eluant was diluted to 10 ml with phosphate-buffered saline and concentrated to 0.5 ml by ultrafiltration with an Amicon YM-5 filter. Finally, these affinity-purified antibodies were reacted with 50kDa and 72kDa zones on another nitrocellulose blot using a dot-immunobinding assay [11].

Staphylococcal V8 Peptide Mapping of 50kDa and 72kDa Transglutaminase

Peptide maps were performed on 50kDa and 72kDa bands from SDS-PAGE as described by Cleveland et al [13]. Gel slices were exposed to 5–500 ng *Staphylococcus aureus* V8 protease during electrophoresis through a 4-cm stacking gel. An additional 30-min incubation without current was included just prior to entry of bromphenol blue into separating gel. Gels were stained with Coomassie blue following electrophoresis.

Activation of Transglutaminase

Extracts in neutral buffer were preincubated in the presence of 0.1 mg/ml of a variety of proteases at 37°C. At intervals from 0–120 min, 25 μl of the preincubation mixture was removed and assayed for transglutaminase activity as previously described [6]. In addition, another 25 μl of preincubation mixture was added to an equal volume of concentrated (2×) Laemmli [9] sample buffer for SDS-PAGE. Control samples were preincubated under the same conditions without added enzyme. Similar activation studies were performed with trypsin, chymotrypsin, plasminogen activator, and plasmin. Activation with dithiothreitol (DTT) and heating were performed as previously described [5].

High-Pressure Liquid Chromatography

High-pressure liquid chromatography, (HPLC), was performed with Waters 721 programmable system controller, 730 data module, 510 and 6000A pumps, and a 441 detector at 280 nm. Proteins were separated using Protein Pak DEAE 5PW (10 μm) column (7.5 mm × 7.5 cm) (Waters, Milford, Massachusetts) eluted at 1 ml/min with a 30 ml linear gradient of 0–0.5 M NaCl in 20 mM Tris-HCl, pH 8.5. One-milliliter fractions were collected, aliquots (50 μl) assayed for transglutaminase activity and/or activation, and the molecular weights of proteins in active fractions determined by SDS-PAGE as described.

RESULTS

The effect of extraction conditions on the molecular weight of transglutaminase is demonstrated in the immunoblot in Fig 1. When transglutaminase is extracted from human scales in neutral buffer without protease inhibitors, transglutaminase is detected as a single 50kDa band by its specific antibody (Fig 1*A*, *lane 1*). When 0.1 mM PMSF, a serine protease inhibitor, and 1.0 mM EDTA are added to extraction buffer, a second positive staining band appears at 72kDa on immunoblots (Fig 1*A*, *lane 2*). The total activity extracted was twofold higher when PMSF and EDTA were present. The 72kDa band is a doublet or triplet with one to two faint bands seen just below the 72kDa band. No staining was seen when a rabbit nonimmune serum or second antibody alone was used in the immunoblot. Extracts from cultured human keratinocytes and neonatal foreskin epidermis studied under similar conditions had predominantly 72kDa bands on immunoblots (Fig 1*B*).

In order to determine whether the 50kDa and 72kDa proteins contained an identical antigenic determinent, the antibody to each protein was affinity purified as shown in Fig 2. Antibody that bound to 72kDa protein, when eluted from the blot, reacted with both 50kDa and 72kDa proteins. Similarly, antibody that bound to 50kDa protein, when eluted from the blot, reacted with both 50kDa and 72kDa proteins. Control study using whole antitransglutaminase antibody or combined anti-72kDa and anti-50kDa antibodies also was positive on both antigens. A band of 30kDa stained weakly and broadly especially in blots of heavily loaded samples. Antibody eluted from the 50kDa and 72kDa proteins reacted with that protein band (data not shown).

Fig 3 shows the peptide maps of 50kDa and 72kDa proteins digested with *Staphylococcus aureus* V8 protease at various

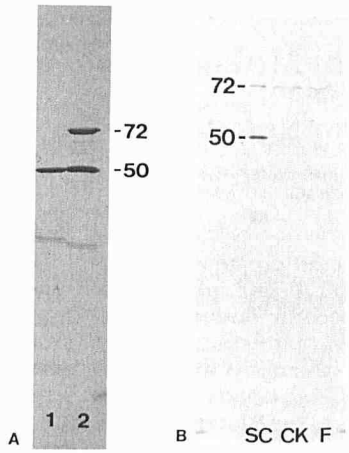


FIG 1. Immunoperoxidase staining of transglutaminase under the different extraction conditions. *A*, Transglutaminases extracted in neutral buffer containing 0.1 M Tris-HCl, pH 8.0, 0.15 M NaCl, with (*lane 2*) or without (*lane 1*) 1 mM EDTA and 0.1 mM PMSF were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and immunoblots were done using rabbit antihuman transglutaminase as described in *Materials and Methods*. Appropriate controls for immunoblots, a rabbit nonimmune serum, or second antibody alone, were performed simultaneously. *B*, Immunoblots of neutral buffer extract stratum corneum from lamellar ichthyosis (*SC*), cultured keratinocytes (*CK*), and neonatal foreskin epidermis (*F*) prepared as described in *Materials and Methods* and stained with antihuman transglutaminase. The numbers *72* and *50* indicate apparent molecular weights (kDa) derived from molecular weight markers as described in *Materials and Methods*.

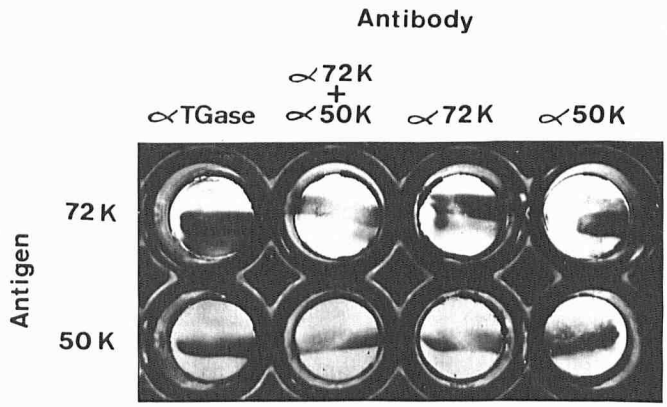


FIG 2. Cross-reactivity of anti-50kDa and anti-72kDa transglutaminase antibody. Specific antibodies, anti-50kDa and anti-72kDa transglutaminase antibody were prepared from whole rabbit antitransglutaminase antisera (αTGase) using affinity purificaton as described in *Materials and Methods*. These affinity-purified antibodies (α50kDa, anti-50kDa; α72kDa, anti-72kDa; α50kDa and α72kDa, anti-50kDa and anti-72kDa mixed) were reacted with 50kDa and 72kDa zones punched out from another nitrocellulose blot using a dot-immunobinding assay.

concentrations. Depending on the concentration of V8 protease, the number of cleavage fragments was increased. Five nanograms caused some digestion, while 25 ng resulted in many fragments of 50kDa, 42kDa, 40kDa, 33kDa, 31kDa, and 25kDa. One hundred twenty-five nanograms and 500 ng produced fragments of the same molecular weights as 25 ng, but in addition, smaller peptides of 23kDa, 22kDa, 19kDa, and 15kDa were seen. With 500 ng, there was no undigested starting material of either 72kDa or 50kDa transglutaminase and both showed digested peptides prominently at 31kDa and 25kDa with some smaller fragments. At all V8 protease concentrations, there were at least 5 identical peptides (3 unequivocally repro-

ducing in photographs) found from digests of 72kDa or 50kDa transglutaminase.

As seen in Fig 4*A*, preincubation with 0.1 mg/ml of trypsin caused a time-dependent enhancement of transglutaminase activity, up to 3 times control values after 2-h incubation. The effect of trypsin on the 50kDa and 72kDa protein staining of immunoblots is seen in Fig 4*B*. At 0 and 15 min both 50kDa and 72kDa bands were present and of about equal intensity. By 30 min and 45 min the intensity of staining of the 72kDa band had progressively diminished with concomitant increase in the intensity of staining of the 50kDa band. Immunologic staining of the 72kDa band was completely eliminated by 60 min and 50kDa showed maximum intensity of stain, which was unchanged at 120 min. At all time points the mobility of 50kDa was unaffected. The presence of a third staining band of about 30kDa is not altered by trypsin.

The effects of plasmin and plasminogen activator were also examined (data not shown). There was no enhancement of transglutaminase activity over a range of concentrations of plasmin from 0.1–0.5 mg/ml or with incubations up to 2 h in duration. Neither was there any alteration in immunologic staining of either 50kDa or 72kDa bands. In contrast, 0.1 mg/ml chymotrypsin decreased the activity of transglutaminase over control with time. Silver-stained gels and immunoblots of enzyme-incubated samples failed to show the presence of either 50kDa or 72kDa bands (data not shown).

The neutral buffer extract was chromatographed on DEAE columns using HPLC (Fig 5). The 72kDa enzyme reproducibly eluted at 0.18 M NaCl as determined by enzyme assays, SDS-PAGE, and immunoblots. There was a 10-fold increase in enzyme specific activity in the fraction containing the 72kDa transglutaminase. This fraction was activated by heating in the presence of calcium and by trypsin (Table I). After heating there was a 16-fold increase of activity. Trypsin (0.1 mg/ml) activated 72kDa transglutaminase 2.7-fold above control values. A 50kDa ETG was not found in HPLC eluates.

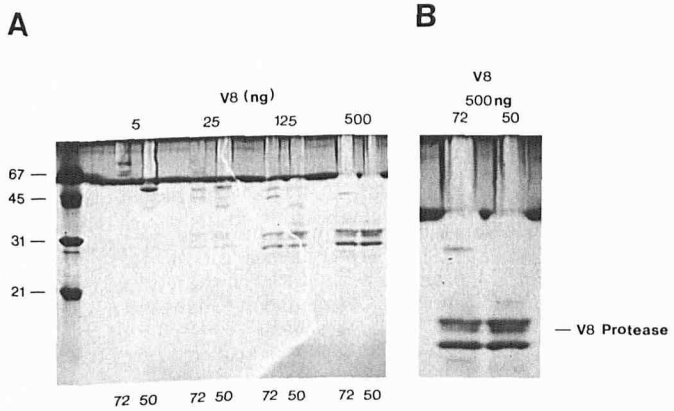
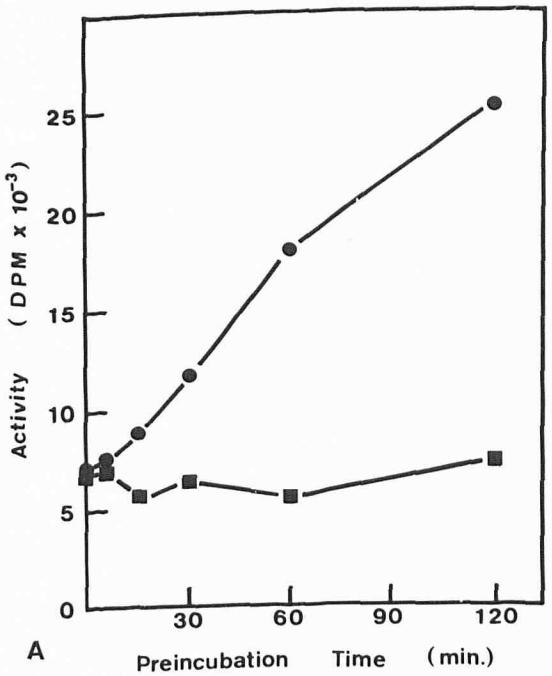


FIG 3. *A*, Peptide maps of 50kDa and 72kDa transglutaminase digested with *Staphylococcus aureus* V8 protease. Stratum corneum extracts in neutral buffer containing 1 mM EDTA and 0.1 mM PMSF were separated on SDS-PAGE. Gel slices of 50kDa and 72kDa bands (as indicated) were applied to a second SDS-PAGE (15% acrylamide) in the presence of varying amounts (25, 125, 500 ng) of *Staphylococcus aureus* V8 protease as indicated; electrophoresis was carried out as described in *Materials and Methods*. Molecular weight markers and their apparent molecular weights are in the leftmost gel lane and on the left side of the gel respectively. *B*, Digestion patterns of 50kDa and 72kDa bands with 500 ng of *Staphylococcus aureus* V8 protease enlarged from the left panel. Peptide fragments with the same molecular weights were observed. The numbers *72* and *50* refer to the molecular weight of the starting material.

DISCUSSION

The inclusion of protease inhibitors in our normal extraction buffers has allowed us to detect a second form of human ETG. The extreme sensitivity of the 72kDa form of ETG to proteolysis prevented detection of this enzyme in our previous studies. A serine protease, trypsin, is capable of converting the 72kDa form of the enzyme to the 50kDa form. Other proteases, plasmin, and plasminogen activator had no effect while chymotrypsin, under the conditions used, destroyed both forms of the enzyme.

The 72kDa and 50kDa forms of ETG were immunologically cross-reactive with affinity-purified antibodies (Fig 2); peptide maps confirmed the close biochemical relationship of the ETGs (Fig 3). It is possible that conversion between the two forms of ETG is responsible for some of the increases in activity during normal epidermal differentiation [4], after treatment of mouse



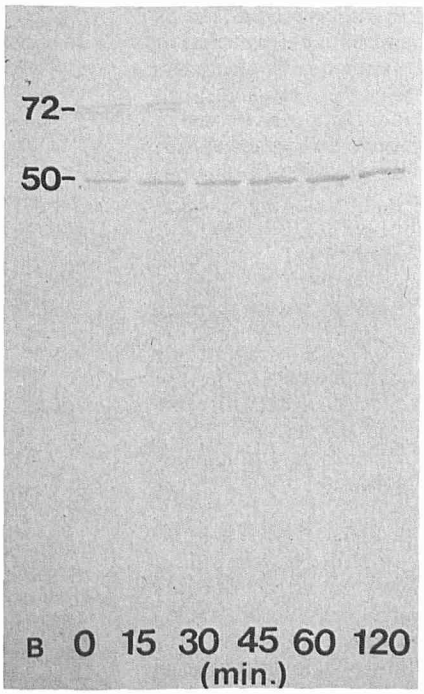


FIG 4. *A*, Activation of transglutaminase by trypsin. Extracts in neutral buffer containing 1 mM EDTA and 0.1 mM PMSF (25 μl) were preincubated in a final volume of 50 μl at 37°C in the presence of 0.1 mg/ml trypsin. At the indicated time intervals, 25 μl of the preincubation mixture was removed and assayed for transglutaminase activity (●) as described in *Materials and Methods*. Control samples (■) were preincubated under the same conditions without added trypsin. *B*, Immunoblots with antitransglutaminase after trypsin incubation. At the indicated time intervals (*min.*), after trypsinization, equal volume of concentrated (2×) Laemmli sample buffer was added to the trypsin transglutaminase reaction mixture and heated at 100°C for 5 min. Twenty microliters of each sample was applied on SDS-PAGE and electrophoretically transferred to nitrocellulose. Immunoblots were performed using rabbit antihuman transglutaminase as described in *Materials and Methods*.

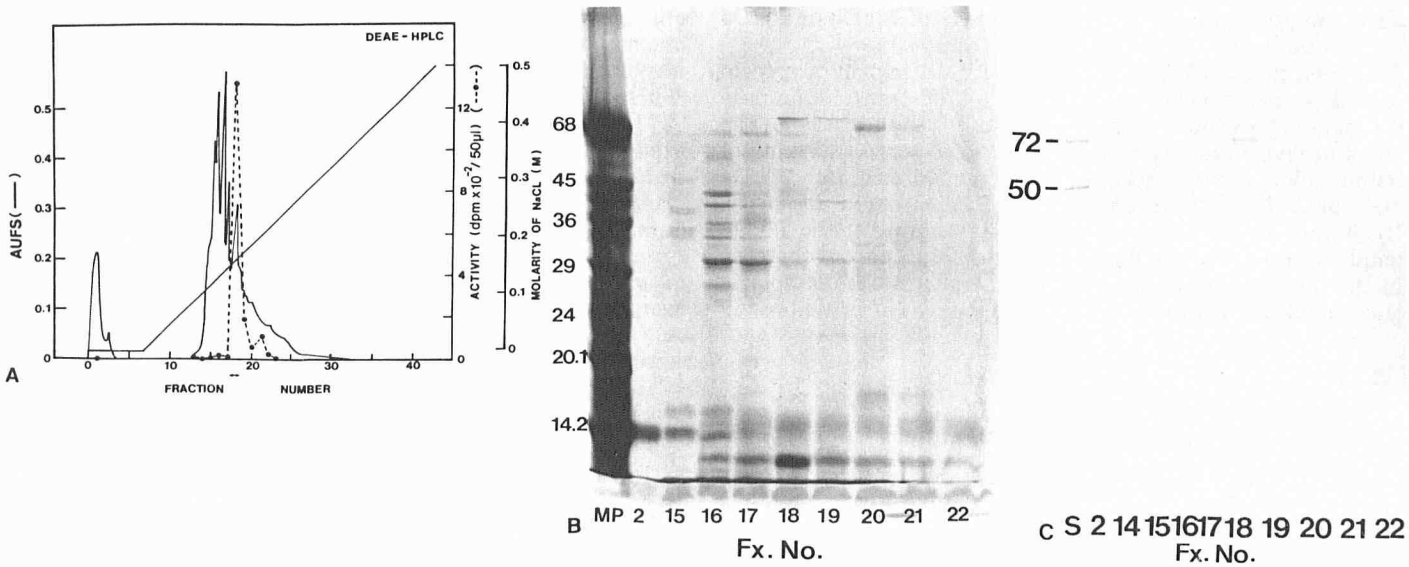


FIG 5. DEAE fractionation of human ETG. *A*, Transglutaminase activity (--●--); AUFS (absorbance units full scale) (——); and NaCl molarity (——). Chromatography as outlined in *Materials and Methods*. *B*, Silver stained SDS-polyacrylamide gels of HPLC fractions corresponding to those in panel *A*. Marker proteins (*MP*) with apparent molecular weight in kilodaltons as indicated on the left hand side of the gel; HPLC fractions as indicated. Highest enzyme activity was in fraction 18 which has a 72kDa protein. *C*, Immunoblot stained with antihuman transglutaminase. The panel and fractions correspond to the protein stain in *B*.

TABLE I. *Effect of heating and trypsin on 72kDa transglutaminase activity*

	dpm/50 µl	% of control
Fraction with 72kDa ETG	659	100
+ Ca^{++}, DTT, 56°C	10,682	1621
+ Trypsin (0.1 mg/ml)	1,770	269

HPLC fraction contained only 72kDa ETG by immunoblot analysis. Activation conditions were as previously described [1,5,6].

keratinocytes with tumor promoters [14,15], and retinoid treatment [8,15]. Since transglutaminase from neonatal foreskin epidermis and from human epidermal keratinocytes contains predominantly the 72kDa protein (Fig 1), we do not regard the state of enzyme to be specifically related to the nature of ichthyotic stratum corneum. Thacher and Rice have reported multiple forms of transglutaminase in extracts of cultured squamous cell carcinoma [16,17] and have suggested different roles for the enzyme forms relative to the state of differentiation of the cells. The relationship of those transglutaminases to the enzymes in this report is unknown.

It is possible that the two forms of transglutaminase have a precursor–product relationship such as exists during activation of plasma factor XIII [18,19]. Activation by organic solvents, heating, or limited proteolytic digestion was associated with the disappearance of protein staining and immunoreactivity of the 72kDa tranglutaminase and with increased immunoreactivity of the 50kDa ETG (Fig 4, and data not shown for organic solvent activation). A fraction containing only 72kDa activity could be isolated using HPLC. In this fraction the enzyme could be activated by heating to 56°C or with trypsin. Our previous studies show comparable enhancement of enzyme activity of preparation with almost entirely 50kDa protein after similar pretreatments [5,6]. Although the 50kDa and 72kDa forms of transglutaminase are immunologically and biochemically related, the exact nature of the relationship will require more detailed studies.

S. Thacher and R. Rice (Cell 40:685–695, 1985), have described a particulate transglutaminase solubilized with Emulgen from culture keratinocytes with a molecular weight of 92kD by immunoprecipitation. Monoclonal antibodies to that transglutaminase did not immunoprecipitate transglutaminase activity in the neutral buffer extract. The relationship of the transglutaminase to the 72kD transglutaminase in this report is under study in our laboratory.

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