LUNG MATRIX-ASSOCIATED TRANSGLUTAMINASE: CHARACTERIZATION AND ACTIVATION WITH SULFHYDRYLS*

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Transglutaminases (EC 2.3.2.13) are enzymes that catalyze a calcium-dependent acyl transfer reaction in which the γ-carboxamidopyridine of peptide-bound glutamine residues, acting as acyl donors, and the primary amino groups in a variety of compounds (including peptide-bound lysine) acting as acyl acceptors, react to form monosubstituted γ-amides of peptide-bound glutamic acid. These enzymes are widely distributed in both cells and extracellular compartments in higher animals. Different transglutaminases promote specific biological events. The polymerization of fibrin during hemostasis (for review, see Ref. 1), for example, is catalyzed by plasma Factor XIIIa. This transglutaminase, formed from thezymogen Factor XIII by thrombin action, is distinct from the transglutaminase that catalyzes the formation of the envelope of the stratum corneum during terminal differentiation of keratinocytes (2). A soluble transglutaminase found in all cells, the activity of this enzyme appears to decrease during cellular proliferation and malignant transformation (4). However, its physiological role is not known.

Previous reports (3, 5) indicate that the majority of the enzyme termed tissue transglutaminase is located in the cytosol. However, this may be an oversimplification; the tissue distribution of transglutaminase may well be organ-specific. We report here that the majority of transglutaminase in rat lung is found in the particulate fraction, and that more than one species of transglutaminase was found in this organ. The lung is in direct contact with the external environment and its physiological functions are closely related to its overall cell-matrix organization (for review, see Ref. 6). Human lung has been found to contain the highest level of transglutaminase activity/weight of tissue (7). In order to understand the functional role of this enzyme in lung, it was considered necessary to first examine both the enzyme and the matrix with which it is associated. Here we report the results of this examination. The findings provide a basis for some speculation concerning the manner in which the enzyme is maintained in insoluble form and its activity is regulated in lung.

MATERIALS AND METHODS

Extraction of Transglutaminase from Tissue—Adult rat whole organs (supplied frozen from Pel-Freez Biologicals, Rogers, AR) were perfused with cold phosphate-buffered saline, cut into small pieces, and homogenized separately in 5 volumes of 0.05 M Tris acetate buffer, pH 7.5, containing 0.25 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM benzamidine-HCl, and 0.1 mM chymostatin (Buffer A) with the use of a Brinkmann Polytron homogenizer 5-10 times for 10 s each on setting 8. The homogenates were centrifuged at 33,000 × g for 30 min at 4 °C. The pellets were washed twice with Buffer A at 4 °C. The resultant supernatant—henceforth called second extract—was split into two equal volumes, with one containing 0.5 M NaCl. After pooling (and saving) the 33,000 × g supernatant—henceforth called first “high salt” extract—the residual pellet was rehomogenized in Buffer A (without the high salt concentration) then centrifuged at 33,000 × g for 30 min at 4 °C. The resultant supernatant—henceforth called second extract—was split into two equal volumes, with one

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subsequently being treated with 20 mM DTT for several hours at 4 °C and the other remaining untreated. The resultant pellet was treated overnight with 20 mM DTT in Buffer A with gentle stirring at 4 °C and the suspension subsequently centrifuged to obtain the 33,000 × g supernatant—henceforth called third extract.

A separate batch of Buffer A (containing 0.5 M NaCl) extracted rat lung residual pellet was treated (five times) for 2 h at 4 °C with a combination of chaotropic agent (50 mM KSCN) and sulfhydryl reagent (20 mM DTT) in Buffer A. The extracts were pooled, the protein was precipitated out with 50% saturated ammonium sulfate, and the resultant precipitate redissolved in a small aliquot of Buffer A.

The different extracts were subsequently analyzed by both ion-exchange chromatography and gel filtration. DEAE-Sephacel (Pharmacia, Uppsala, Sweden) and Bio-Gel A-5m (Bio-Rad) columns were, respectively, used to separate the enzymes.

Transglutaminase Assay—A slightly modified transglutaminase assay method (9) was used to determine the transglutaminase activity of the various fractions. It involves measurement of the amount of [1,4-14C]putrescine incorporated into the protein acceptor, dimethylated casein. The 0.5-mL standard reaction mixtures contained 1% dimethylated casein, 0.1 M Tris acetate, 1 mM EDTA, 10 mM CaCl₂, 1% Lubrol PX, 4 mM DTT, 0.5 μCi of [1,4-14C]putrescine (107.1 mCi/mmol, New England Nuclear) at pH 7.5, and 20–50 μL of enzyme fraction. The reactions were carried out at 37 °C for 1 h and were terminated by addition of 4 ml of 7.5% cold trichloroacetic acid. After 30 min in the cold, the trichloroacetic acid-insoluble precipitates were filtered on GF/A glass filters (Whatman) and washed with about 20 ml of 5% cold trichloroacetic acid. The filters were dried and transferred to scintillation counting vials. Ten ml of scintillation counting fluid, Hydrofluor (National Diagnostics, Somerville, NJ), was added, and the vials were vigorously shaken to pulverize the filters. The vials were counted in a scintillation counter, and the transglutaminase activity of the fractions expressed as cpm/h/aliquot volume.

Non-lipid Macromolecular Component Analysis of Rat Lung Residual Pellet—The glycosaminoglycan content of the high salt extracted residual pellet was determined by measuring uronic acid by the method of Burton (15). The elastin content was determined by the method of Briscoe and Loring (14) and the DNA content by the method of Burton (15).

RESULTS

Cellular and Subcellular Distribution of Transglutaminase—Examination of the transglutaminase distribution in the organs (muscle, lung, liver) and nonnucleated blood cells, i.e. erythrocytes and platelets, and cultured Chinese hamster ovary cells reveals a pattern as shown in Table I. Here it is seen that the cultured Chinese hamster ovary cells and erythrocytes have the bulk of transglutaminase activity associated with the soluble fraction, whereas with muscle and lung the majority of transglutaminase activity is found in association with the particulate fraction. The liver, on the other hand, whose cells are not involved in a significant association with extensive filamentous structure or extracellular matrix, contains the bulk of its transglutaminase activity in soluble form. This suggested to us that the transglutaminase activity distribution may be a reflection of the cellular organization. The results given in Table II provide evidence that soluble transglutaminase activity in cells is associated with the cytosol (142,000 × g supernatant) fraction.

Solubilization of Rat Lung Residual Pellet Transglutaminase—To determine whether the particulate-bound transglutaminase activity in the rat lung is bound to cell membrane, the rat lung residual pellet was extracted with n-octyl-β-D-glucopyranoside at various detergent concentrations. As shown in Fig. 1, treatment of the pellet with this detergent...
TABLE III
Extraction of transglutaminase activity from rat lung pellet

The rat lung residual pellet activities here ranged between 85 and 90% of the total lung tissue homogenate activities. The pellets were suspended with the various extracting solutions at a final protein concentration of 5 mg/ml. Protein was determined by the method of Lowry et al. (32). The suspensions were stirred gently at 4 °C for 1 h. Following centrifugation at 33,000 × g for 30 min, the supernatant from each sample was collected and the transglutaminase activity assayed in each. In the case of thrombin, the suspension was centrifuged after 30 min incubation of pellet with thrombin at 37 °C.

<table>
<thead>
<tr>
<th>Extracting solution</th>
<th>Transglutaminase activity (activity extracted from pellet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer alone</td>
<td>7.5</td>
</tr>
<tr>
<td>Buffer + 0.15 M KCl</td>
<td>12.0</td>
</tr>
<tr>
<td>Thrombin (20 units)</td>
<td>9.4</td>
</tr>
<tr>
<td>n-Octyl-β-D-glucopyranoside (0.5%)</td>
<td>11.0</td>
</tr>
<tr>
<td>Triton X-100 (0.5%)</td>
<td>10.3</td>
</tr>
<tr>
<td>Zwittergent 3-12 (0.5%)</td>
<td>9.0</td>
</tr>
<tr>
<td>CHAPS (0.5%)</td>
<td>10.5</td>
</tr>
<tr>
<td>Lubrol PX (0.5%)</td>
<td>10.5</td>
</tr>
<tr>
<td>KSCN (50 mM)</td>
<td>8.6</td>
</tr>
<tr>
<td>KSCN (125 mM)</td>
<td>24.6</td>
</tr>
<tr>
<td>KSCN (250 mM)</td>
<td>19.0</td>
</tr>
<tr>
<td>KSCN (500 mM)</td>
<td>17.4</td>
</tr>
<tr>
<td>Dithiothreitol (50 mM)</td>
<td>140.9</td>
</tr>
<tr>
<td>Dithiothreitol (20 mM), KSCN (50 mM)</td>
<td>327.3</td>
</tr>
</tbody>
</table>

solubilized only a small fraction of the transglutaminase activity. In the presence of 1.5% detergent, the particulate-bound enzyme activity was found to be decreased. The percent activity solubilized with this concentration of detergent was no more than that solubilized (~12%) by treatment of the pellet with Buffer A containing 0.15 M KCl (Table III). In order to determine whether this detergent was solubilizing membrane proteins, the activity of a plasma membrane marker, 5′-nucleotidase, was measured. As shown in Fig. 1, n-octyl-β-D-glucopyranoside at final concentrations of 0.5 and 1.5% (values near or greater than the critical micelle concentration of 0.73 for this detergent) efficiently extracted 5′-nucleotidase activity from the pellet. Four other detergents, Triton X-100, Zwittergent 3-12, CHAPS, and Lubrol PX, failed also to solubilize rat lung pellet transglutaminase activity (Table III). As with the n-octyl-β-D-glucopyranoside, these ionic and nonionic detergents extracted only about 10% of the particulate-bound transglutaminase activity.

Limited proteolysis of the pellet matrix with thrombin failed to release or solubilize a significant portion of the transglutaminase activity (Table III). The per cent transglutaminase activity solubilized (9.4%) with this treatment was not significantly different from that solubilized with the detergents.

The chaotropic agent KSCN provided a degree more solubilization of transglutaminase activity from rat lung pellet. As shown in Table III, treatment of the pellet at 4 °C for 1 h with 125 mM KSCN in Buffer A resulted in a 2–3-fold increase in transglutaminase activity extracted relative to that treated only with Buffer A. However, when higher concentrations of KSCN were employed, no more efficient extraction was obtained.

The 40% of glycerol in Buffer A, which has been used for extraction of cellular contractile proteins (16), did not affect the solubilization of particulate-associated transglutaminase.

Effect of Sulfhydryl Agent on Transglutaminase—The pellet was also treated with 20 mM DTT in Buffer A for 1 h at 4 °C. The transglutaminase activity in the extract of the DTT-treated pellet was increased 18-fold over that from pellet treated with Buffer A alone. It seems clear from these findings that treatment with dithiothreitol caused activation of transglutaminase. This was verified by treatment of enzyme that had been extracted from the pellet with Buffer A alone with dithiothreitol. This treatment resulted in a 5.5-fold increase in enzymatic activity (Fig. 2A). In order to determine whether treatment with dithiothreitol also provided a more efficient extraction of enzyme from the pellet, we measured the transglutaminase activity remaining after extraction with this agent. The results are shown in Fig. 2B. It is apparent that about 62% of the Buffer A-extracted residual pellet transglutaminase activity remained associated with the pellet after five further extractions with Buffer A, whereas about 17% remained associated with the pellet after five extractions with dithiothreitol in Buffer A. When the pellet was extracted with a solution of both DTT and KSCN in Buffer A, the transglutaminase activity of the extract was increased to about 44 times that of the Buffer A extract.

Non-lipid Macromolecular Component Analysis of Rat Lung Residual Pellet—The high salt-extracted residual pellet was found to consist of a proteoglycan-rich matrix (Table IV). It is apparent that homogenization of rat lung in Buffer A containing 0.5 M NaCl results in a greater extraction of

![Fig. 2. Activation of cytosol transglutaminase and extraction of transglutaminase from rat lung pellet with dithiothreitol. Panel A depicts cytosol transglutaminase activation with 20 mM dithiothreitol (□), the original cytosol transglutaminase activity designated as 100%; (□), the cytosol transglutaminase activity after dithiothreitol activation). Panel B depicts the remaining transglutaminase activity associated with residual pellet after 5 extractions (□, the original pellet transglutaminase activity designated at 100%; ○, the pellet transglutaminase activity remaining after five extractions). The extractions were done in Buffer A with or without 20 mM dithiothreitol. The resuspended activity of the 20 mM dithiothreitol-treated pellet transglutaminase before the first 33,000 × g centrifugation was 5.8-fold greater than that of the untreated pellet.](image-url)
was repeated four times. The macromolecular components were thus determined in the residual pellet after five extractions.

An increase in activity. Chromatography of this DTT-treated transglutaminase B,4 was eluted at a NaCl concentration of 0.18 M. That in the second peak, transglutaminase C, eluted from a DEAE-Sephacel column at the ionic strength 0.28 M of the organ. Although elastin in the extracted pellets was found to be higher than collagen or DNA, it represents less than one-third of the original concentration (estimation based on proportion to the concentration of glycosaminoglycan before and after extraction). In order to determine the predominant glycosaminoglycan form of the proteoglycan fraction, we made a comparison between the uronic acid elution profiles on Sepharose 6B columns of chemically treated and enzymatically treated pellet glycosaminoglycan. As shown in Fig. 3, treatment of a sample of pellet glycosaminoglycan with chondroitinase ABC, an enzyme specific for the degradation of chondroitin sulfates and unreactive toward heparin or heparin sulfate, failed to shift the uronic acid elution profile to the right (the lower molecular weight range), whereas treatment with nitrous acid, specific for the cleavage of heparin, was successful in shifting the uronic acid elution profile to the right of that of untreated sample. These observations, and the fact that pellet glycosaminoglycan during its purification was eluted from a DEAE-Sephacel column at the ionic strength specific for heparin sulfate elution (data not shown), suggest that the predominant glycosaminoglycan moiety of the proteoglycan fraction of the rat lung residual pellet is in the heparin sulfate form.

Ion-exchange Chromatography of Lung Transglutaminase—The 33,000 x g supernatant (or first high salt extract) obtained from homogenized rat lungs displayed (after dialysis against Buffer A) two peaks of transglutaminase activity when chromatographed on DEAE-Sephacel using a linear salt gradient (Fig. 4A). The enzyme in the first peak, designated transglutaminase B,4 was eluted at a NaCl concentration of 0.18 M. That in the second peak, transglutaminase C, eluted at 0.28 M NaCl. Transglutaminase C was completely extracted by Buffer A, i.e. none of this enzyme was found in later extracts, as is evident in the chromatogram obtained with the second extract of the pellet (Fig. 4B). The relative distribution of transglutaminases in the supernatant fraction was estimated as 60% B and 40% C enzyme. Thus, the total amount of transglutaminase C present in the lung homogenate amounted to less than 10% of total transglutaminases. Treatment of transglutaminase B with 20 mM DTT caused a 6-fold increase in activity. Chromatography of this DTT-treated enzyme showed a new peak of activity at 0.23 M NaCl (Fig. 4C). This new material, termed transglutaminase B1, was the sole component of a 20 mM DTT extract (i.e. third extract) of the pellet (Fig. 4D).

When the well-characterized guinea pig liver transglutaminase (7) was chromatographed as outlined in Fig. 4, it displayed a single peak of activity identical to that of lung transglutaminase C. Its chromatographic position was unchanged by its treatment with dithiothreitol. Also, this guinea pig liver enzyme showed no change in degree of activity following dithiothreitol treatment. These findings suggest that lung transglutaminase B is a matrix-bound enzyme and that it is structurally and catalytically distinct from the so-called tissue enzyme, i.e. the soluble transglutaminase C.

Gel Filtration—Transglutaminase B from the 33,000 x g fraction of lung homogenate and that from the subsequent (or second) extract of the residual pellet (Fig. 5, A-I and B, respectively) eluted from a gel-filtration column at the same position, designated position 1 (Kda 0.64). Transglutaminase C was eluted from this column later (Fig. 5, A-II) with a Kda value of 0.70 (position 2). Transglutaminase B1, from 20 mM DTT treatment of transglutaminase B, also eluted at position 2 (Fig. 5C), as did the bulk of the transglutaminase activity from the pellet treated with 20 mM DTT (Fig. 5D). A small portion of this DTT-extracted activity eluted at a third position (position 3) of Kda 0.83. Enzyme extracted from the pellet with 20 mM DTT, 50 mM KSCN eluted from the column at positions 2 and 3 in about equal proportions (Fig. 5E).

For convenience of differentiating against the well-characterized guinea pig liver enzyme (transglutaminase C), we have designated this species as transglutaminase B.
We have observed that blood cells and cultured Chinese hamster ovary cells and organs, such as liver, that are devoid of significant association with extensive filamentous structure or extracellular matrix have the majority of their transglutaminase activity in a soluble form, whereas organs that contain extensive filamentous structure (muscle) or extracellular matrix (lung) have much of their transglutaminase activity in a particulate form. This observation prompted us to question whether different species of transglutaminase exist and if so, it is unclear whether different growth characteristics of cell in culture have significant effect on the expression of multiple forms of transglutaminase. As to this latter point, it has been found that considerable transglutaminase activity and transglutaminase antigen is associated with detergent-insoluble material in human lung fibroblasts which are confluent and arrested in culture (21).

In our studies, transglutaminase B from rat lung was eluted from the gel filtration column with a $K_D$ value similar to those of 100,000-dalton proteins and transglutaminase C was eluted with a $K_D$ value similar to those of 80,000-dalton proteins and guinea pig liver transglutaminase. In this respect, rat lung transglutaminase B is similar to rat chondrosarcoma transglutaminase and liver particulate-associated transglutaminase (see accompanying manuscript (36)). Since there was a fair amount of erythrocytes trapped in the lung, it is likely that most of the transglutaminase C found in the supernatant fraction of the lung homogenate may have been contributed by the red blood cell which contains transglutaminase that was shown to have similar molecular properties to that of the transglutaminase C (23, 24).

When cytosol (or second extract) transglutaminase B was treated with dithiothreitol, it was converted into one (transglutaminase B1) having the same affinity for the anion-exchanger and the same Stokes radius as those found associated with the particulate-bound transglutaminase. Concomitant with the conversion, this treatment resulted in the enzyme being activated 6-fold. Transglutaminase C was not affected by dithiothreitol treatment (data not shown). Since the cytosol fraction was found to contain 60% transglutaminase B and 40% transglutaminase C, the DTT-induced 5.5-fold

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**DISCUSSION**

**G.P. liver transglutaminase**, guinea pig liver transglutaminase.

**FIG. 4. Anion-exchange chromatography of rat lung extracts.** The extracts were applied on a 4.2 × 12-cm column of DEAE-Sephacel resin and eluted with a 1900-ml linear salt gradient from 0 to 0.5 M NaCl in Buffer A. Fractions of 20 ml were collected and assayed for transglutaminase activity. The extracts applied to this column were previously chromatographed on DEAE-Sephacel. The fractions denoted by the horizontal bars in Fig. 4 were pooled, and the protein was precipitated with 50% saturated ammonium sulfate and redissolved in Buffer A before application. A-I, transglutaminase B from the first high salt extract; A-II, transglutaminase C from the first high salt extract; B, transglutaminase B from the second extract of the residual pellet; C, transglutaminase B1, derived from 20 mM DTT treatment of transglutaminase B; D, transglutaminase B1, derived from residual pellet treated with 20 mM DTT (i.e. third extract); E, transglutaminase activity derived from a separate batch of Buffer A (containing 0.5 M NaCl)-extracted residual pellet after treatment with 20 mM DTT, 50 mM KSCN (this extract was not chromatographed on DEAE-Sephacel prior to elution on the gel-filtration column).

The extracts were applied on a 2.6 × 90.7-cm column of Buffer A-equilibrated Bio-Gel A-5m, and the collected fractions were assayed for transglutaminase activity. The extracts applied to this column were previously chromatographed on DEAE-Sephacel. The fractions denoted by the horizontal bars in Fig. 4 were pooled, and the protein was precipitated with 50% saturated ammonium sulfate and redissolved in Buffer A before application. A-I, transglutaminase B from the first high salt extract; A-II, transglutaminase C from the first high salt extract; B, transglutaminase B from the second extract of the residual pellet; C, transglutaminase B1, derived from 20 mM DTT treatment of transglutaminase B; D, transglutaminase B1, derived from residual pellet treated with 20 mM DTT (i.e. third extract); E, transglutaminase activity derived from a separate batch of Buffer A (containing 0.5 M NaCl)-extracted residual pellet after treatment with 20 mM DTT, 50 mM KSCN (this extract was not chromatographed on DEAE-Sephacel prior to elution on the gel-filtration column).

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8 Although "cytosol" here is being used to refer to the actual 33,000 × g supernatant obtained this term will suffice since only an insignificant fraction (≤1.5%) of transglutaminase activity could be further pelleted at 142,000 × g (the established centrifugal force for obtaining cytosol).
activation of transglutaminase activity in the cytosol fraction (Fig. 2) represented a 10-fold activation of transglutaminase B in the cytosol.

This conversion may also represent a mechanism that might be occurring in vivo, as suggested by Bures and Goldsmith (25); namely, that an insoluble-soluble translocation mechanism may be a means of compartmentalizing or controlling transglutaminase activity in vivo. Both the increase in catalytic activity and extractability of bound transglutaminase with dithiothreitol treatment, as seen in our studies, would certainly lend support to such a hypothesis. The fact that the major portion of transglutaminase B was found to be in a non-activated state in the homogenate suggests that either such an activation mechanism occurs in a way that is proportionally small to distinguish, or the activated enzyme is rapidly inactivated. In fact, the sulfhydryl agent-activated enzyme was found to be very labile during the storage. Sulfhydryl-dependent activation of enzyme activity has been observed with human leucocyte collagenase (26).

Treatment of the particulate fraction of the rat lung with chaotropic agent partially enhanced the extractability of particulate-associated transglutaminase but did not affect the enzyme activity. Our previous report (27) that all species of lung transglutaminase cross-react with polyclonal and monoclonal specific anti-guinea pig liver transglutaminase suggests that transglutaminases B and C retain common antigenic sites. Studies to establish the genetic origin of the transglutaminases are currently in progress.

Our finding of differential distribution of transglutaminase activity, the observations that a small fraction of transglutaminase is in the serum-free medium of cultured tumor cells (28), and finally, the reports of others (29, 30) that exocytosis of intracellular enzymes occurs as an active cellular process, all suggest a possible function for the matrix-bound transglutaminase. That is that this enzyme, which is structurally and catalytically different from the soluble enzyme, may, in some manner, regulate the overall cell-matrix organization or morphogenesis of higher multicellular organisms. Our findings that the predominant form of transglutaminase is particulate in organs which display kinetic behavior, i.e., contractility as in muscle and elasticity as in lung, also lead us to speculate whether these enzymes are involved in cell-cell or cell-matrix stabilization. More specifically, it would be important to know whether these enzymes play a role in the fusion of the many separate cells that form the single, unusually large muscle cell (for review, see Ref. 31) or in the maintenance and organization of the extracellular matrix that can make up approximately 25% of the dry weight of the entire lung (6).

REFERENCES
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