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Purification and characterization of a cytosolic transglutaminase from a cultured human tumour-cell line

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Transglutaminases are a family of Ca^{2+}-dependent enzymes that catalyse the formation of isopeptide bonds between the side chains of glutamine and lysine residues. The enzymes have been hypothesized to be involved in a wide range of cellular processes, including growth and differentiation and stabilization of the cytoskeleton. The human epidermal carcinoma-cell line, A431 cells, have relatively high amounts of a cytosolic transglutaminase activity that varies upon treatment of the cells with epidermal growth factor. We demonstrate here that this cytosolic activity has the biochemical and immunological properties of a tissue transglutaminase. We also report the purification of this enzyme to apparent homogeneity by a protocol which involves a novel affinity-elution step. Polyclonal antibodies to the transglutaminase were raised and used to identify the enzyme by Western blotting. The availability of purified transglutaminase and anti-transglutaminase antibodies will permit further study of the role of this enzyme in the growth of this hormone-responsive human tumour-cell line.

INTRODUCTION

Transglutaminases are a family of Ca^{2+}-dependent enzymes that catalyse the incorporation of primary amines into protein-bound glutamine residues via an acyl-transfer reaction (Folk & Finlayson, 1977; Folk *et al.*, 1980). When a protein-bound lysine residue serves as the primary amine donor, the reaction results in the formation of an ϵ-(γ-glutamyl)-lysine isopeptide bond that serves to cross-link proteins (Folk & Finlayson, 1977; Lorand & Conrad, 1984; Birckbichler *et al.*, 1973, 1978).

Transglutaminases have been isolated and studied from various sources (Folk, 1980). In certain differentiated cell types, specialized forms of the enzyme have been found to cross-link proteins, resulting in the formation of distinctive structures. For example, epidermal transglutaminase is responsible for the formation of cornified envelopes in differentiated keratinocytes (Rice & Green, 1977, 1979; Rothnagel & Rogers, 1984). Hair-follicle transglutaminase is thought to be responsible for the cross-linking of hair shaft proteins, rendering them insoluble (Chung & Folk, 1972; Harding & Rogers, 1972). A widespread form of the enzyme, tissue transglutaminase, has been identified in a number of mammalian cell types (Connellan *et al.*, 1971; Abe *et al.*, 1977; Folk, 1980), in both non-differentiated and differentiated cells (Maddox & Haddox, 1985; Byrd & Lichti, 1987). The biological role of tissue transglutaminase is not well understood, but evidence has suggested its involvement in a number of activities, including cell growth and differentiation (Birckbichler & Patterson, 1978; Murtaugh *et al.*, 1983), cytoskeletal stabilization (Maccioni & Arechaga, 1986) and intracellular vesicle transport (Bungay *et al.*, 1986; Gomis *et al.*, 1986).

We have been investigating the transglutaminase activity present in homogenates prepared from A431 cells. A431 cells are a human epidermal carcinoma-cell line (Fabricant *et al.* 1977). They possess high numbers of EGF receptors (Wrann & Fox, 1979), and respond well to the hormone when short-term responses are studied. However, A431 cells have been shown to cease proliferation when cultured continuously in the presence of EGF (Gill & Lazar, 1981). Interestingly, culture of these cells with low concentrations of EGF also leads to an increase in the specific activity of a cytosolic transglutaminase (Rosdy *et al.*, 1986). To understand better the role of transglutaminase in the growth of A431 cells, it was necessary to characterize the type of transglutaminase present in these cells. We demonstrate here that the transglutaminase present in A431-cell cytosol exhibits the biochemical and immunological properties of a tissue transglutaminase. We also describe a rapid procedure for the purification of this soluble transglutaminase from A431 cells which utilizes a novel affinity-elution step.

EXPERIMENTAL

Materials

[1,4(n)-^{3}H]Putrescine dihydrochloride was purchased from Amersham. Mouse monoclonal antibodies CUB74 and CUB11 were generously supplied by Dr. Paul Birckbichler (Noble Foundation, Ardmore, OK, U.S.A.). Goat anti-mouse and goat anti-rabbit IgG alkaline-phosphatase-conjugated second antibodies were from Accurate Chemical and Scientific Corp., Westbury, NY, U.S.A. Alkaline phosphatase colour reagents Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were from Promega. Bicinchoninic acid protein assay

Abbreviations used: EGF, epidermal growth factor; HBSS, Hanks balanced salts solution.

reagents were from Pierce. Silver-staining reagents for SDS/polyacrylamide gels were from Bio-Rad. All other reagents were obtained from Sigma.

Preparation of A431-cell cytosol

Lysates were prepared essentially as described previously (Dadabay & Pike, 1987). Briefly, cultures of A431 cells in 150 mm-diam. plates were washed with ice-cold HBSS and scraped into 15 ml of the same buffer. Cells were pelleted by low-speed centrifugation. The resulting cell pellet was resuspended in 0.5 ml of buffer containing 5 mM-Tris/HCl, pH 7.4, 250 mM-sucrose, 0.2 mM-$MgSO_4$, 1 μg of leupeptin/ml, 10 mM-benzamidine, 1 mM-phenylmethanesulphonyl fluoride and 5 μg of α_2-macroglobulin/ml and homogenized with a small Dounce homogenizer. The lysate was centrifuged for 30 min at 100000 *g*. The supernatant was used as cell cytosol.

Purification of A431-cell soluble transglutaminase

A431 cells were grown to confluence in five roller bottles in media consisting of Dulbecco's modified Eagle's medium containing 10% (v/v) fetal-bovine serum. The growth medium was removed and the cultures were washed with cold HBSS. The cells from each roller bottle were then scraped into a total of 200 ml of buffer containing 50 mM-Tris/HCl, pH 7.5, 1 mM-EGTA, 250 mM-sucrose, 10 mM-dithiothreitol, 1 mM-phenylmethanesulphonyl fluoride, 1 μg of leupeptin/ml and 10 mM-benzamidine. The cells were lysed with 20 strokes of a Dounce homogenizer. The lysate was centrifuged at 100000 *g* for 30 min, and the supernatant was collected and stored at −70 °C. All subsequent chromatographic steps were carried out at 4 °C.

The cytosolic fraction (approx. 650 mg of protein) was batch-adsorbed on to 8 ml of Whatman DE52 DEAE-cellulose with gentle stirring in a beaker for 2 h. The resin was then collected in a Buchner funnel and rinsed with an additional 150 ml of buffer A (50 mM-Tris, pH 7.5, 1 mM-EGTA, 0.5 mM-dithiothreitol, 1 mM-phenylmethanesulphonyl fluoride, 1 μg of leupeptin/ml and 10 mM-benzamidine). The DE52 resin was then transferred into a 16 mm-internal-diam. glass chromatography column and washed with 25 ml of buffer A containing 0.1 M-KCl. The column was eluted with a linear gradient of 0.1–0.5 M-KCl in buffer A (80 ml total). Fractions were collected and assayed for transglutaminase activity. Fractions containing transglutaminase activity were pooled, adjusted to 10% (v/v) glycerol, and stored at −20 °C overnight.

The DE52 pool was dialysed against buffer B (50 mM-Hepes, pH 7.0, 1 mM-EGTA, 0.5 mM-dithiothreitol, 1 mM-phenylmethanesulphonyl fluoride, 1 μg of leupeptin/ml and 10 mM-benzamidine) for 3 h. The resulting material was applied to a 5 ml heparin–agarose column and washed with 25 ml of buffer B containing 20 mM-NaCl. Transglutaminase activity was eluted with 25 ml of buffer B containing 1 mM-GTP and 20 mM-NaCl.

The heparin–agarose eluate was supplemented with 5 mM-$CaCl_2$, and applied to a 7 ml α-casein–agarose column (Croall & DeMartino, 1986). The column was washed with 30 ml of buffer A containing 5 mM-$CaCl_2$ and 1 M-NaCl. Transglutaminase activity was eluted with 25 ml of buffer A containing 5 mM-EGTA and 0.5 M-NaCl. This eluate was concentrated to 1 ml with a Pharmacia 10 ml Omegacell ultrafiltration kit containing an M_r-10000 cut-off membrane.

Transglutamine assay

The transglutaminase activity of samples was determined by assaying the incorporation of [^{3}H]putrescine into *NN*-dimethylated casein as described previously (Dadabay & Pike, 1987; Birckbichler *et al.*, 1976), with the following modifications. The assay was stopped by addition of EGTA to a final concentration of 15 mM. Protein-bound [^{3}H]putrescine and free [^{3}H]putrescine were separated by desalting the reaction mixtures over 5 mm × 105 mm columns containing Sephadex G-50. The buffer used for desalting contained 50 mM-Tris, pH 8.0, 50 mM-NaCl, 15 mM-EGTA and 0.02% NaN_3 (buffer C). *NN*-Dimethylated casein containing bound [^{3}H]putrescine was eluted from the columns in the void volume of 1.6 ml. This material was collected directly into 20 ml plastic vials and processed for liquid-scintillation counting. The columns were regenerated by washing with 5 column vol. of buffer C.

Lactate dehydrogenase assay

Lactate dehydrogenase was assayed as described previously (Fritz, 1965).

Protein determination

Protein concentrations were determined by the Pierce bicinchoninic acid assay. When dithiothreitol was present in the samples, proteins were first precipitated in 0.0125% deoxycholate and 6% (w/v) trichloroacetic acid, and the protein concentration was determined by the method of Lowry *et al.* (1951).

Gel electrophoresis and staining

SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970). Gels were stained for protein with a Bio-Rad silver-staining kit.

Production of polyclonal antibodies

One 5 kg male New Zealand White rabbit was injected subcutaneously with 20 μg of the purified A431-cell transglutaminase in Freund's complete adjuvant. The rabbit was boosted twice at 1-month intervals with Freund's incomplete adjuvant containing approx. 20 μg of purified protein in the first boost and approx. 10 μg in the second boost. Serum was collected 10 days after each boost, and was analysed for reactivity with the A431-cell transglutaminase by slot-blot analysis. Detection was accomplished with alkaline-phosphatase-conjugated goat anti-rabbit IgG second antibody (see below).

Western blotting

Protein samples were run on SDS/polyacrylamide gels as described above and then transferred to nitrocellulose with a Hoeffer Transblot apparatus. The nitrocellulose was blocked by incubation with 3% (w/v) bovine serum albumin in TBST (50 mM-Tris, pH 7.9, 150 mM-NaCl and 0.05% Tween-20). Primary antibody [CUB74 anti-(guinea-pig liver transglutaminase) monoclonal antibody or rabbit anti-(A431-cell transglutaminase) antisera] was added and the incubation continued for 2 h at room temperature. After the nitrocellulose had been washed three times in TBST, alkaline-phosphatase-conjugated second antibody was added at 1:5000 dilution in TBST for 30 min. After washing, immunoreactive proteins were detected by development with Promega alkaline phosphatase colour reagents.

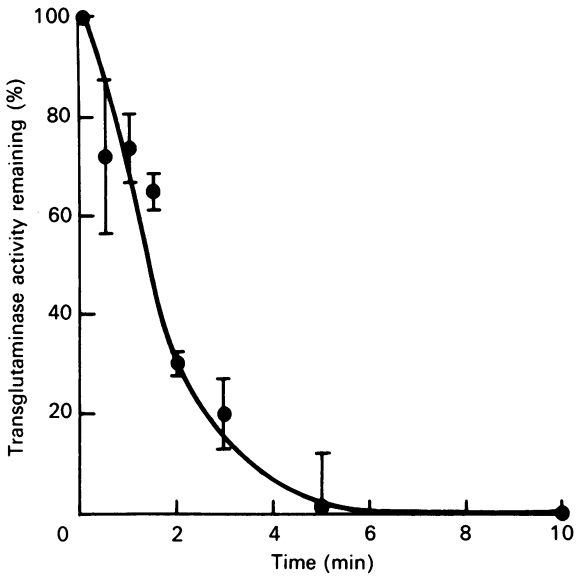

Fig. 1. Thermal inactivation of soluble transglutaminase from A431 cells

Equal samples of the 100000 *g* soluble fraction prepared from A431-cell homogenates were incubated for the indicated times at pH 9.0 at 37 °C. The samples were then assayed for transglutaminase activity by the method described in the Experimental section.

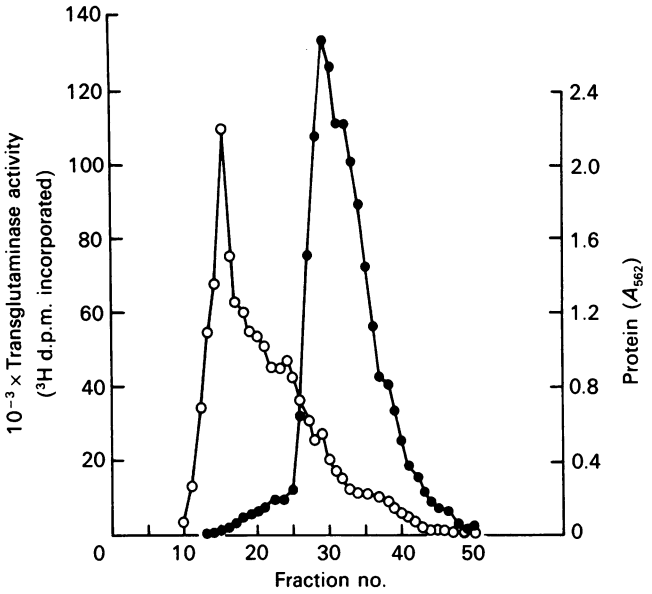

Fig. 2. Chromatography of the A431-cell cytosol on DE52 DEAE-cellulose

The 100000 *g* supernatant from A431-cell homogenates was loaded batchwise on to 8 ml of DE52 DEAE-cellulose, washed and eluted with a linear 0–0.5 M-KCl gradient as described in the Experimental section. A total of 50 fractions was collected and assayed for transglutaminase activity (●) and protein (○).

RESULTS

A431 cells are a human epidermal carcinoma-cell line; thus they could contain epidermal transglutaminase as well as the widespread tissue transglutaminase. Since tissue and epidermal transglutaminases can be distinguished on the basis of their biochemical properties, we carried out a preliminary investigation of the biological properties of the A431-cell transglutaminase. Lichti *et al.* (1985) have demonstrated that the activity of tissue transglutaminase was labile to incubation at pH 9 at 37 °C, whereas the epidermal transglutaminase was stable to this treatment. As shown in Fig. 1, incubation of A431-cell cytosol at pH 9 at 37 °C led to the rapid and complete inactivation of the transglutaminase activity present in the fraction. The half-life of the enzyme under these conditions was 1.5–2 min. The inactivation of the transglutaminase was due to the elevated temperature, as incubation of cytosol for 20 min at pH 9 on ice failed to cause a loss of enzyme activity. These findings suggest that the soluble A431-cell transglutaminase is a tissue transglutaminase and that it represents the major, if not the only, transglutaminase in the cytosol.

Tissue-type transglutaminase can also be distinguished from epidermal transglutaminase, as the former is inhibited by GTP whereas the latter is unaffected (Schmidt *et al.*, 1988). The A431-cell transglutaminase activity was markedly inhibited by GTP, with 50% inhibition at approx. 200 μM (results not shown). This response to GTP is characteristic of tissue, but not epidermal, transglutaminase, again suggesting that the major enzyme in A431-cell cytosol is tissue transglutaminase.

To establish more definitively the nature of the A431-cell enzyme, the soluble transglutaminase present in A431 cells was purified to apparent homogeneity. The starting material for the purification was the 100000 *g* cytosolic fraction prepared from A431 cells grown in roller bottles. The initial purification step was chromatography on DE52 DEAE-cellulose. Nearly all (95–97%) of the transglutaminase activity present in the soluble fraction of A431 cells bound to the anion-exchange resin and was eluted in a single peak between 0.2 M- and 0.3 M-KCl, as shown in Fig. 2.

The pooled fractions from the DE52 column were further purified by adsorption to a heparin–agarose column, followed by elution with a low-ionic-strength buffer containing GTP. As shown in Fig. 3, most of the transglutaminase bound to the heparin–agarose column and was not released by washing with low-ionic-strength buffer. However, inclusion of GTP in this buffer effectively released the transglutaminase from the heparin–agarose. By this procedure, a significant amount of transglutaminase activity was recovered in the GTP eluate, whereas most of the protein was present in the column flow-through and wash. The purification achieved by this step averaged 9-fold, but was as high as 17-fold in some preparations.

The final step in the purification exploits the Ca^{2+}-dependent binding of transglutaminase to its substrate, casein. The pool of transglutaminase eluted from the heparin–agarose column with GTP was supplemented with 5 mM-Ca^{2+} and loaded on an α-casein–agarose column. The amount of transglutaminase that adsorbed to this column ranged from 60 to 90% in different experiments. After a high-salt wash which removed much of the contaminating protein, transglutaminase activity was eluted from the column with 5 mM-EGTA. The eluate was immediately concentrated by ultrafiltration, since dilute solutions of the purified enzyme were unstable to storage at 4 °C. The final product was stored in

50 mM-Tris (pH 7.5)/1 mM-EGTA/0.5 mM-dithiothreitol and was stable at 4 °C for up to 2 weeks.

A purification table for the preparation of A431-cell transglutaminase is shown in Table 1. The major loss occurred during the α-casein–agarose step. This was in part due to the presence of a portion of the transglutaminase activity in the flow-through of this column. The loss of transglutaminase enzyme may also result from the ability of the transglutaminase to catalyse the attachment of itself to the column resin or to other proteins via the formation of isopeptide bonds. This type of catalytic activity is especially likely to occur during loading and washing of the α-casein–agarose column, since Ca^{2+} is present during these steps.

Fig. 4 shows a silver-stained SDS/polyacrylamide gel of a sample of the starting material, DE52 pool, heparin–agarose pool and casein–agarose pool. The most highly purified casein–agarose fraction exhibits a single protein band with an M_r of 83000. This size falls well within the range of sizes reported for tissue transglutaminase from a variety of sources (Folk, 1980). As shown in Fig. 4, the transglutaminase band was greatly enhanced after the heparin–agarose GTP affinity-elution step.

The most highly purified transglutaminase was used as antigen for the production of polyclonal antibodies in rabbits. Antiserum obtained from a rabbit inoculated with the A431-cell transglutaminase recognized the purified protein on Western blots, as shown in Fig. 5, lane *b*. Pre-immune serum from this rabbit did not detect the

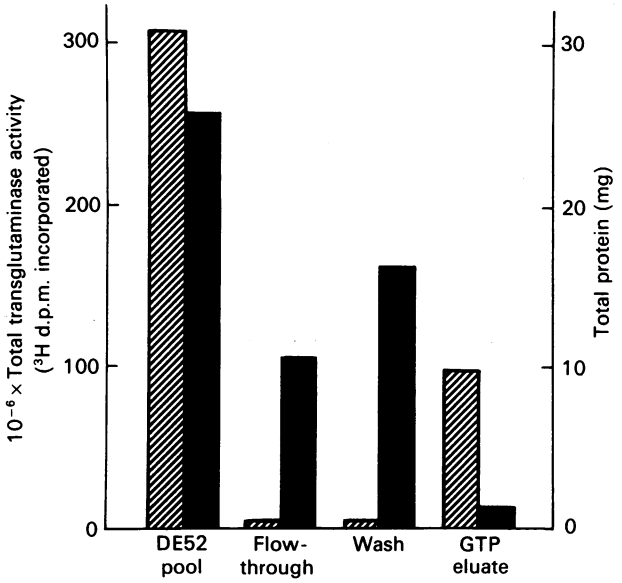

Fig. 3. Affinity elution of transglutaminase activity from heparin–agarose

The pool of transglutaminase activity from the DE52 step was loaded on to heparin–agarose and washed with 20 mM-NaCl in buffer B. The column was then eluted with 1 mM-GTP in buffer B with 20 mM-NaCl as described in the Experimental section. Bars show total transglutaminase activity (▨) and protein content (■) recovered in each fraction. Values shown represent means of duplicate determinations.

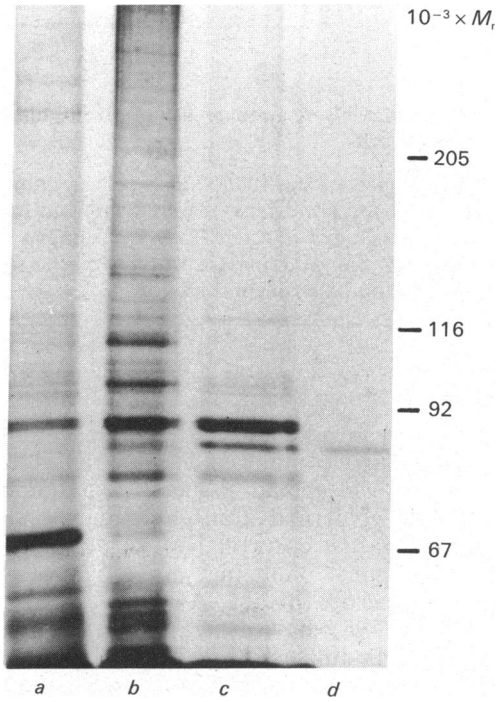

Fig. 4. SDS/polyacrylamide-gel electrophoresis of A431-cell cytosolic transglutaminase

Samples of the 100000 *g* starting material (lane *a*), the DE52 pool (lane *b*), the GTP eluate from heparin–agarose (lane *c*) and the EGTA eluate from casein–agarose (lane *d*) were run on an SDS/8%-polyacrylamide gel. The gel was fixed and silver-stained to detect the proteins.

Table 1. Purification of A431-cell cytosolic transglutaminase

Transglutaminase was purified from A431-cell cytosol as described in the Experimental section. Values for activity and protein content represent averages of duplicate determinations.

Step	Protein (mg)	Sp. activity (nmol/min per mg)	Yield (%)	Purification (fold)
Cytosol	650	0.18	100	—
DE52	55	2.0	90	11
Heparin–agarose	3.3	32	90	177
Casein–agarose	0.15	156	20	890

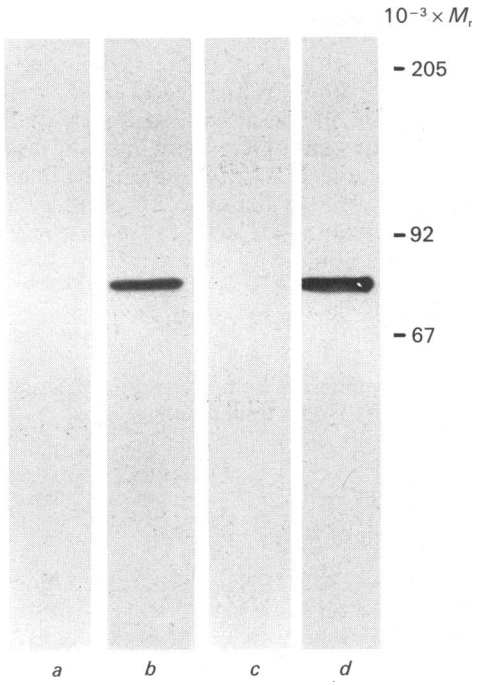

Fig. 5. Western blot of purified A431-cell cytosolic transglutaminase

Samples of the most highly purified A431-cell soluble transglutaminase were subjected to SDS/polyacrylamide-gel electrophoresis, followed by electrophoretic transfer to nitrocellulose paper. The paper was cut to obtain individual lanes, and the lanes were made to react with: lane *a*, pre-immune rabbit antiserum; lane *b*, polyclonal rabbit antiserum to A431-cell transglutaminase; lane *c*, non-reactive mouse monoclonal antibody; lane *d*, mouse monoclonal antibody raised against guinea-pig liver tissue transglutaminase. After washing and blocking, the strips were made to react with the appropriate second antibody conjugated to alkaline phosphatase. Immunoreactive proteins were detected as described in the Experimental section.

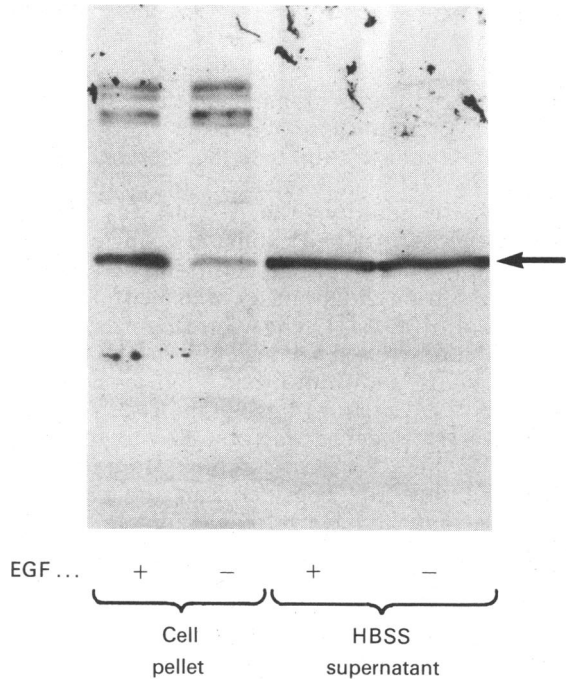

Fig. 6. Western blot of transglutaminase in A431-cell lysates

A431 cells were treated with or without EGF for 30 min, and then harvested by scraping into HBSS. After a low-speed centrifugation to pellet the cells, proteins in the HBSS were precipitated with trichloroacetic acid. The cell pellets were lysed as described in the Experimental section, and samples of the precipitated HBSS supernatants and cell pellets were analysed on an SDS/8%-polyacrylamide gel. After electrophoretic transfer of the proteins to nitrocellulose, the transglutaminase was detected by Western blotting with the mouse monoclonal antibody.

human transglutaminase (lane *a*). The purified human transglutaminase was also found to cross-react with a monoclonal antibody raised against guinea-pig liver tissue transglutaminase (lane *d*), but not with a control non-reactive monoclonal antibody (lane *c*). These findings indicate that the A431-cell transglutaminase possesses the antigenic properties of a tissue transglutaminase.

We have observed previously (Dadabay & Pike, 1987) that elevated transglutaminase activity was associated with the cell pellets of A431 cultures that had been treated with EGF as compared with pellets derived from control cultures. Using the monoclonal antibodies to detect the A431-cell tissue transglutaminase, we investigated the basis for this phenomenon. Cultures of A431 cells were treated with or without 33 nM-EGF for 20 min and then washed with ice-cold HBSS. The cells were scraped into 15 ml of HBSS and pelleted by low-speed centrifugation for 2 min. The HBSS was removed and 10% trichloroacetic acid added to precipitate any proteins present. The cell pellets were then homogenized in buffer A. Samples of the protein precipitated from the HBSS and the cell pellets were then analysed for transglutaminase by Western-blotting analysis. The results are shown in Fig. 6. Surprisingly, a large proportion of the tissue transglutaminase from the A431 cells was present in the HBSS into which the cells had been scraped. Taking into account the different percentages of the total HBSS and cell-pellet fractions loaded on the gel, it could be estimated that approx. 90% of the soluble transglutaminase was present in the HBSS supernatant. Only about 10% of the activity was associated with the cell pellet. However, by both Western blotting and activity assay (results not shown), more transglutaminase was associated with the cell pellets from EGF-treated cultures.

To determine whether this preferential retention of enzyme in the cell pellets of EGF-treated cells was specific for the transglutaminase, the activity of lactate dehydrogenase, another cytosolic enzyme, was assessed in the cell pellets from control and EGF-treated cultures. Both lactate dehydrogenase and transglutaminase activities were greater in the cell pellets derived from EGF-treated cells as compared with control cells. Furthermore, the increase in transglutaminase activity was essentially identical with that in lactate dehydrogenase activity (229% and 233% respectively). These data suggest that A431 cells release their cytosolic components when harvested mechanically. Treatment with EGF appears to ameliorate this effect somewhat, thereby preserving more cytosolic enzymes in the cell pellet.

DISCUSSION

We have found that the biochemical and antigenic properties of the transglutaminase activity in A431-cell cytosol correspond to those of a tissue transglutaminase. The transglutaminase exhibited the thermolability and inhibition by GTP characteristic of a tissue transglutaminase. In addition, the enzyme was purified to apparent homogeneity and exhibited a single band with an M_r of approx. 83000, again consistent with the size of known tissue transglutaminases. Furthermore, the enzyme purified from A431 cells was recognized by monoclonal antibodies raised against guinea-pig liver tissue transglutaminase, indicating that it possessed the antigenic properties of a tissue transglutaminase. Throughout these studies no evidence was obtained to suggest that A431 cells contained a second soluble transglutaminase activity. We therefore consider that the tissue transglutaminase that we have purified represents the main source of soluble transglutaminase activity in A431 cells.

The purification procedure reported here incorporates a novel affinity-elution step that was based on the observed inhibition of the enzyme by GTP, as well as the ability of the transglutaminase to bind weakly to heparin–agarose. It is possible that binding of GTP to the transglutaminase alters its conformation enough to induce release of the enzyme from the heparin–agarose. Alternatively, the negatively charged GTP may directly compete with the sulphate groups on the heparin to effect elution. In any case, this step appears to introduce some specificity into the heparin–agarose step and results in a significant purification of the enzyme.

We had initially attempted to purify the transglutaminase by chromatography on Reactive Red–agarose (Croall & DeMartino, 1986) in place of the heparin–agarose column. However, we found that the Red–agarose step resulted in large losses of activity and destabilization of our enzyme. By replacing this step with the heparin–agarose GTP affinity elution, recovery and stability of the enzyme were greatly improved. Our novel procedure was rapid. The entire purification could be accomplished in 2 days. The relatively high yield makes the procedure suitable for purification of the enzyme from tissue-culture sources where only limited quantities of starting material are available.

Polyclonal antibodies to this purified human tissue transglutaminase were raised and were found to be useful for detecting the protein on Western blots. Using these antibodies as well as a monoclonal antibody raised against the guinea-pig liver tissue transglutaminase, we were able to quantify transglutaminase antigen in lysates of A431 cells. The finding that A431 cells release cytosolic components, including the transglutaminase, after mechanical harvesting and that short-term treatment with EGF alters this tendency was unexpected. EGF has been shown to induce membrane ruffling and cellular retraction in A431 cells (Chinkers *et al.*, 1979). It is possible that these effects of EGF on the cytoskeleton are the basis for the apparent differential stability of the cells during scraping.

A431 cells are a widely used model system for the study of the mechanism of action of EGF (Carpenter & Cohen, 1979). These cells have also been employed in studies of the differentiation of tumour-derived keratinocytes, that at some level involves the activity of a transglutaminase catalysing the formation of cornified envelopes (Rosdy *et al.*, 1986). The finding that long-term culture with EGF affects the levels of transglutaminase in these cells (Rosdy *et al.*, 1986) and also affects cell growth and differentiation (King & Sartorelli, 1986) suggests that this system may be useful for unravelling the role of transglutaminases in programmed cell growth. The availability of purified tissue transglutaminase from A431 cells as well as polyclonal antibodies to this ubiquitous enzyme will permit us to study further the role of tissue transglutaminase in the growth of this hormone-responsive tumour cell.

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