Bovine aortic endothelial cell transglutaminase

Enzyme characterization and regulation of activity

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Bovine aortic endothelial cells contain Ca^{2+} -dependent tissue-type transglutaminase. Its activity in these cells was high, with apparent K_m and V_{max} values with respect to putrescine of 0.203 mM and 18.5 nmol/min per mg of protein, and its activity was inhibited by the three competitive inhibitors dansylcadaverine, spermine and methylamine. The molecular mass of endothelial cell transglutaminase estimated by gel filtration chromatography was 88 kDa and it was immunoprecipitated by rabbit monospecific antiserum raised against rat liver transglutaminase. Its enzymic activity rose when the cell cultures reached confluence, and was further increased when their proliferation was arrested (synchronized at G_0/G_1 phase). Most of the enzymic activity was found in the 15000 g soluble fraction, with only 4–22 % of the activity found in the particulate fraction, depending on the state of cell proliferation. Examination of these cellular fractions by SDS/polyacrylamide-gel electrophoresis and immunoblotting revealed that at confluence endothelial cells have accumulated transglutaminase antigen in their 15000 g particulate fraction. A series of experiments demonstrated the existence of a latent transglutaminase form in non-proliferating cells, and suggested that this might involve the formation of an inhibitory complex. Treatment of cell lysates and the 15000 g particulate fraction with high salt concentration showed a significant increase in transglutaminase activity. Mixing experiments using the 100000 g particulate fraction or purified rat liver transglutaminase on one hand and the cytosolic fraction on the other showed dose-dependent inhibition of the transglutaminase activity of the latter. It is concluded that endothelial cells contain a particulate fraction-residing inhibitor of transglutaminase which interacts via ionic interaction with the enzyme.

INTRODUCTION

Transglutaminase (EC 2.3.2.13; protein-glutamine: amine- γ -glutamyltransferase) catalyses Ca^{2+} -dependent acyl transfer reactions between γ -carboxamide groups of peptide-bound glutamine residues, which serve as the acyl donors, and various primary amines, resulting in new γ -amides of glutamic acid and ammonia (Folk & Finleyson, 1977; Folk, 1980; Lorand & Conrad, 1984). The most common acyl acceptor substrates involve ϵ -amino groups of peptide-bound lysine residues and the primary amino groups of polyamines. The involvement of the former acceptor groups results in the formation of ϵ -(γ -glutamyl)lysine bonds, which are found in a wide variety of cells and tissues (Pisano *et al.*, 1968; McKee *et al.*, 1970; Folk & Finleyson, 1977; Birckbichler *et al.*, 1978). The precise biological role(s) of cellular transglutaminase remain(s), however, unclear (Folk, 1980; Lorand & Conrad, 1984). It has been suggested that transglutaminase is involved in the regulation of cell growth (Birckbichler & Patterson, 1978; Milhaud *et al.*, 1980; Birckbichler *et al.*, 1981; Korner & Bachrach, 1985, 1987) and cell differentiation (Rice & Green, 1979; Murtaugh *et al.*, 1983; Davies *et al.*, 1985; Maddox & Haddox, 1985; Mehta & Lopez-Berestein, 1986). Its activity varies considerably depending on the state of cell proliferation. It increases with decreasing cell growth

(Birckbichler & Patterson, 1978; Milhaud *et al.*, 1980) and decreases in virally- or chemically-transformed cells (Birckbichler *et al.*, 1977; Birckbichler & Patterson, 1978). Indeed, in normal and virally-transformed fibroblasts, a good correlation was found between transglutaminase activity and the amount of ϵ -(γ -glutamyl)lysine bonds formed (Birckbichler *et al.*, 1978). Thus, there appears to be an inverse relationship between cell growth on the one hand and cellular transglutaminase activity and transglutaminase-dependent product formation on the other (Birckbichler *et al.*, 1978), although the identity and biological role of these products have not been determined. Other factors that have been shown to increase cellular transglutaminase activity include treatment with sodium butyrate (Byrd & Lichti, 1987; Lee *et al.*, 1987), serum (Scott & Russell, 1982; Korner & Bachrach, 1985), β -adrenergic receptor agonists (Korner & Bachrach, 1985, 1987), guanosine 5'-phosphate (Achyuthan & Greenberg, 1987) and retinoids (Kojima *et al.*, 1987).

Transglutaminases have been described in a variety of tissues, cells and body fluids (Pisano *et al.*, 1968; Folk & Finleyson, 1977; Rice & Green, 1979). These include liver, epidermal, hair-follicle, macrophage and prostate transglutaminases, and platelet, placental and plasma Factor XIII (Folk & Finleyson, 1977; Folk, 1980; Lorand & Conrad, 1984), and most recently endothelial

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; aFGF, acidic fibroblast growth factor; PBS, phosphate-buffered saline; DTT, dithiothreitol; TED, Tris/EDTA/DTT buffer; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis.

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cell transglutaminase (Greenberg *et al.*, 1987; Kojima *et al.*, 1987). Vascular endothelial cells represents a slowly growing population of cells which normally turns over at a low rate (Schwartz & Benditt, 1976). However, a variety of physiological and pathogenic stimuli can injure the endothelial lining, resulting in denudation of cells and subsequent re-endothelialization of the denuded area (Gimbrone *et al.*, 1974). These cells play an important role in maintaining the non-thrombogenic characteristics of the vascular lining and are thought to play an important defensive role against the development of atherosclerosis (Mustard *et al.*, 1982; Ross, 1986). Therefore, the purpose of the present investigation was to characterize further the biochemistry of vascular endothelial cell transglutaminase, and to investigate the effects of different proliferative states of these cells on its activity.

EXPERIMENTAL

Chemicals

[2,3-³H]Putrescine dihydrochloride (17.4 Ci/mmol) and carrier-free Na¹²⁵I were purchased from New England Nuclear (Boston, MA, U.S.A.). *N,N*-Dimethylated casein was from Calbiochem-Behring Corp. (San Diego, CA, U.S.A.). Rabbit immunoglobulin (IgG) to human Factor VIII was obtained from Accurate Chemicals (Westbury, NY, U.S.A.). Tissue culture dishes were from Costar Co. (Cambridge, MA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), antibiotics, glutamine and trypsin were obtained from Grand Island Biological Company (Grand Island, NY, U.S.A.). Acidic fibroblast growth factor (aFGF) was generously donated by Dr. D. Gospodarowicz (University of California Medical Center, San Francisco, CA, U.S.A.). Nitrocellulose sheets BA-85 were from Schleicher and Schuell (Keene, NH, U.S.A.). Gel filtration media, electrophoresis and electroblotting equipment were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Affinity-purified fluorescein isothiocyanate (FITC)-conjugated IgG against rabbit IgG, goat anti-rabbit IgG (affinity purified), molecular mass protein standards for gel filtration and SDS gel electrophoresis, and all other chemical reagents, which were of analytical grade, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mono-methylated and di-methylated dansylcadaverine were synthesized from dansylcadaverine as previously described (Krishnamurthy, 1982). The chemical identity of these compounds was verified by elemental analysis; both were > 99 % pure as determined by t.l.c.

Cell culture procedures

Endothelial cells were obtained from fresh bovine aortas, which were first washed several times in sterile 20 mM-phosphate-buffered saline (PBS; 137 mM-NaCl/2.7 mM-KCl/4.3 mM-Na₂HPO₄/1.5 mM-KH₂PO₄, pH 7.4). The cells were scraped off the luminal side of the vessel into 60 mm tissue culture plates containing DMEM supplemented with 10 % FCS, 2 mM-glutamine and 200 units each of penicillin and streptomycin per litre of medium, and incubated in a humidified atmosphere of air/CO₂ (19:1) at 37 °C. On the following day, the medium was replaced with fresh medium, and every other day 50 ng of aFGF/ml was added. After 3–5 days, several cell colonies could be identified, each containing 5–15 cells. Individual cell colonies exhibiting typical

endothelial cell patterns were transferred after treatment with 0.5 % trypsin and 0.02 % EDTA in PBS to 35 mm dishes using small glass rings. After the cells had reached confluence, they were maintained in the same medium for an additional 4–6 days. The cultures were examined carefully under light microscopy, and only those cultures in which typical endothelial cell morphology was observed (Gospodarowicz *et al.*, 1977), involving confluent monolayers of polygonal, closely apposed cells with non-overlapping 'cobblestone' morphology, underwent further passages. These cell cultures exhibited positive staining for Factor VIII-related antigen. For the various experiments the cells were seeded in 60 mm dishes, at a density of 5×10^3 cells/cm²; under these conditions the cells reached 50 % confluence in 3–4 days, and 100 % confluence in 8–10 days. For some experiments confluent cultures were synchronized by serum depletion, which involved replacing the medium with fresh DMEM supplemented with 0.2 % FCS for 48 h. Under these conditions 90–95 % of the cells are growth-arrested at the G₀/G₁ phase of the cell cycle (Pardee, 1974). The experiments described in this report were conducted with cloned endothelial cell cultures between passages 5 to 16.

Preparation of endothelial cell extracts

All steps were carried out on ice or at 4 °C with ice-cold buffers. Three different preparations were made.

Cell lysates. Cell cultures were washed twice with ice-cold PBS, once with 50 mM-Tris/HCl, pH 7.4/1 mM-EDTA/1 mM dithiothreitol (DTT) (TED buffer) supplemented with 150 mM-NaCl (TED buffer saline), and then scraped into 1 ml of TED buffer saline per dish. The cells were sedimented by centrifugation (250 *g* for 5 min at 4 °C), resuspended in TED buffer containing 0.5 mM-phenylmethanesulphonyl fluoride (PMSF) to yield a protein concentration between 1.5 and 2.5 mg/ml. The cells were then lysed by repeated (three times) freezing and thawing. The efficiency of cell disruption was verified by light microscopy and Trypan Blue exclusion staining.

Fractionation at 15000 *g*. Cell lysates prepared as described above were centrifuged at 15000 *g* at 4 °C for 5 min and the supernatants collected. The pellet was resuspended and washed twice by recentrifugation in TED buffer. The final pellet was then resuspended by sonication for 15 s in the same buffer to produce a protein concentration of 1.5–2.5 mg/ml.

Fractionation at 100000 *g*. Cell lysates prepared as described above were centrifuged at 100000 *g* for 1 h at 4 °C. The supernatants were removed and saved. The pellet was resuspended and washed twice by recentrifugation in TED buffer. The final pellet was then resuspended in TED buffer to yield a protein concentration of 1.5–2.5 mg/ml by repeated (three times) sonication for 10 s, with 30 s cooling periods in between. The 100000 *g* pellet fraction was then treated for 2 h at room temperature with DNAase and RNAase (both from bovine pancreas; both 50 µg/ml of cell lysate) made in a final concentration of 0.1 M-Tris/HCl, pH 8.0/0.11 M-NaCl/0.015 M-MgCl₂. The pellet was centrifuged again for 1 h at 100000 *g* and resuspended in a small volume of TED buffer to yield a protein concentration of 1 mg/ml, and dialysed exhaustively against TED buffer saline.

Transglutaminase assay

Transglutaminase activity was assayed by [³H]-

putrescine incorporation into dimethylated casein. The assay was carried out essentially as previously described (Korner & Bachrach, 1985) with minor modifications. The final reaction mixture was composed of 50 mM-Tris/HCl, pH 7.4 (at 37 °C), 10 mM-CaCl₂, 15 mM-DDT (in some cases 4 mM as noted), 0.1 μ Ci/0.5 mM-putrescine, 50 mM-NaCl, 800 μ g of dimethylated casein and 30–200 μ g of protein of the unknown sample. Under these experimental conditions there was a linear relationship between the incorporation of putrescine into casein and the amount of the cell extracts assayed (> 0.5 mg of protein) for up to 15 min. For each activity determination, two different amounts of the unknown sample were tested and duplicate aliquots were removed at 0, 3, 6 and 9 min. The enzyme activity for each unknown sample was then computed from the linear regression curve ($r^2 > 0.98$; coefficient of variation < 10%), and the average transglutaminase activity was expressed as nmol of [³H]putrescine incorporated into acid-insoluble protein/min per mg of cell extract protein. Protein determination was according to the colorimetric method of Bradford (1976) with bovine serum albumin (fraction V) as the standard.

Molecular mass determination

The molecular mass of endothelial cell transglutaminase was determined by size-exclusion chromatography. The 15000 g supernatants of confluent cell lysates were applied to a Bio-Gel A-0.5 m, 200–400 mesh, 1.5 cm $\times$ 95 cm column equilibrated with the elution buffer (25 mM-Tris/HCl, pH 7.4/1 mM-EDTA/1 mM-DDT/125 mM-NaCl/0.1 % Nonidet P-40) employing a flow rate of 15 ml/h. The effluent was collected in 1 ml aliquots which were assayed for transglutaminase activity. The molecular mass of endothelial cell transglutaminase was compared with that of rat liver transglutaminase which was purified to homogeneity as previously described (Korner & Bachrach, 1985). The protein molecular mass standards used were β -amylase (220 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa); Dextran Blue (2000 kDa) was used to determine the void volume.

Immunoprecipitation of transglutaminase

Monospecific rabbit antiserum (Korner & Bachrach, 1985) raised against purified rat liver transglutaminase was employed. Endothelial cell transglutaminase from the gel filtration column was pooled (activity 20 nmol/min per ml). Small (0.1 ml) aliquots were first added to tubes containing final concentrations of 0.2 % bovine serum albumin, 0.4 % Nonidet P-40, 0.4 % deoxycholate and 0.1 % SDS in TED buffer saline. Different volume ratios (total volume 60 μ l) of the transglutaminase rabbit antiserum and rabbit non-immunized serum were then added and the immunoprecipitation carried out at room temperature for 1 h using gentle agitation. Both sera had previously been exhaustively dialysed against a buffer composed of 25 mM-Tris/HCl, pH 7.4, 1 mM-EDTA and 150 mM-NaCl. Immuno-complexes were sedimented by centrifugation (13000 g for 2 min) with 0.1 ml of a crude preparation of *Staphylococcus aureus* containing Protein A, which had previously been washed in TED buffer saline. The supernatants were assayed for transglutaminase activity to determine the extent of immunoprecipitation.

Western blots of transglutaminase in cell lysates

Different cell lysates were mixed in equal volumes with sample buffer [100 mM-DDT/1 mM-EDTA/8 M-urea/3 % (w/v) SDS/50 mM-Tris/HCl, pH 6.8], incubated at 95 °C for 5 min and applied onto a SDS/10 % polyacrylamide-gel electrophoresis (PAGE) minigel (Korner & Bachrach, 1985). The separated proteins were electroblotted on to nitrocellulose paper (at 0.9 A for 1 h). The blot was first saturated with 5 % non-fat milk in PBS followed by 1 % non-immunized goat serum. Transglutaminase antiserum, diluted 1:250, was then added and incubated for 2 h, followed by 1 h incubation with ¹²⁵I-labelled affinity-purified goat anti-rabbit IgG (5 $\times$ 10⁵ c.p.m./ml). Between each incubation the nitrocellulose was washed (four buffer changes) for 1 h with 0.1 % Tween 20 in PBS. All sera were diluted in 0.1 % Tween 20 in PBS. The dried nitrocellulose was autoradiographed at -70 °C using a Kodak Curix-X cassette with intensifier screen and Kodak Curix-X film (Kodak, Rochester, NY, U.S.A.). For quantification, four internal standards of purified rat liver transglutaminase and various amounts of cell lysates were transblotted. The labelled transglutaminase bands on the autoradiograms were examined by a scanning densitometer (Quick Scan, Helena Laboratories, Beaumont, TX, U.S.A.); a linear relationship existed between the amounts of the proteins that were fractionated and the absorbance values read from the densitometer for the transglutaminase bands.

Radiolabelling of proteins

Affinity-purified goat anti-rabbit IgG (0.5 mg) was iodinated by incubation with chloramine-T (50 μ l of 5 mg/ml solution) and 0.4 mCi of Na¹²⁵I in PBS in a total volume of 500 μ l. The reaction was terminated after 30 s by the addition of 250 μ l of sodium metabisulphite (100 mg/ml). Unbound Na¹²⁵I was removed by gel filtration chromatography on a Sephadex G-25 column followed by exhaustive dialysis. The radiolabelled IgG had a specific activity of 4 mCi/mg of protein.

RESULTS

Biochemical characterization of endothelial cell transglutaminase

The activity of the endothelial cell transglutaminase was critically dependent on the Ca²⁺ concentration in the assay mixture, and 50 % of the maximum enzymic activity was achieved at approximately 1.5 mM-Ca²⁺. At a Ca²⁺ concentration lower than 1.25 mM, there was essentially no detectable transglutaminase activity. However, at a Ca²⁺ concentration of 2.5 mM and higher, a maximum plateau of transglutaminase activity was achieved, which remained unchanged up to 12 mM-Ca²⁺. Using size-exclusion Bio-Gel A-0.5 m gel chromatography, the molecular mass of endothelial cell transglutaminase was found to be 88 kDa and that of purified rat liver transglutaminase was 80 kDa. The elution profile of transglutaminase activity from the Bio-Gel A-0.5 m column consisted of a single symmetrical peak of enzyme activity. The enzyme activity was inhibited by the primary amines methylamine, spermine and dansylcadaverine, with IC₅₀ values (concentrations producing 50 % inhibition) of 1.8, 0.17 and 0.0019 mM respectively. While dansylcadaverine is one of the most potent inhibitors of Factor XIII and tissue-type transglutaminase (Lorand

Fig. 1. Immunoprecipitation of endothelial cell transglutaminase activity using transglutaminase rabbit antiserum raised against purified rat liver transglutaminase

The 60 μ l serum portion was made of increasing volumes of the transglutaminase antiserum and decreasing volumes of the non-immunized rabbit serum, e.g. the sample containing 0 μ l of the transglutaminase antiserum contains 60 μ l of the non-immunized serum, and the sample containing 40 μ l of the transglutaminase antiserum contains 20 μ l of the non-immunized serum.

et al., 1968), both mono- and di-methylated dansyl-cadaverine were ineffective as inhibitors of purified rat liver transglutaminase and endothelial cell transglutaminase at concentrations effective for dansyl-cadaverine, and required concentrations higher than 1 mM to produce greater than 20% inhibition. The apparent K_m and V_{max} values for the transamidation reaction with respect to putrescine were determined using 30 μ g protein aliquots from the 15000 g soluble fraction of confluent cell cultures after dialysis against 1000 vol. of TED buffer. Six different concentrations of putrescine were employed, ranging from 0.0625 to 1 mM. The K_m and V_{max} parameters were determined from Lineweaver-Burk double reciprocal plots ($r^2 > 0.99$). K_m averaged 0.203 mM and V_{max} averaged 18.5 nmol/min per mg of protein. Partially purified endothelial cell transglutaminase (Bio-Gel A-0.5 m column) was successfully immunoprecipitated by rabbit monospecific antiserum raised against purified rat liver transglutaminase (Fig. 1), indicating cross-reactivity between these two tissue-type transglutaminases.

Total cellular transglutaminase activity at different proliferative states

The specific activity of transglutaminase in endothelial cell cultures at different states of proliferation and confluence was investigated before and after refeeding the cultures with fresh growth medium supplemented with 10% FCS. Marked variability was observed in its

Fig. 2. Induction of transglutaminase activity in endothelial cell cultures at different states of proliferation

The different cell cultures were 50% confluent, 100% confluent and 100% confluent and synchronized. Time courses of transglutaminase activities were determined in these different cultures after stimulation with DMEM containing 10% FCS. At the indicated times duplicate cultures were removed, the cells harvested and transglutaminase activity and protein concentrations determined.

activity depending on the level of confluence and passage. Proliferating cell cultures at approx. 50% confluence had specific transglutaminase activity of about 2 nmol/min per mg of protein. At confluence, however, the basal transglutaminase activity had doubled, reaching levels of about 4 nmol/min per mg of protein. When confluent cultures were synchronized into G_0/G_1 phase by serum depletion, the transglutaminase activity was again doubled, reaching levels as high as 8 nmol/min per mg of protein. Freshly scraped bovine aortic endothelial cells had transglutaminase activity of 3–5 nmol/min per mg of protein. In general, cells at higher passages had higher transglutaminase activity, of as much as 2–3-fold higher (results not shown). Therefore, each set of experiments was carried out using cells of the same passage.

Upon refeeding these different cultures, significant fluctuations in cellular transglutaminase activity were observed. Fig. 2 is representative of four such experiments carried out in cells of passages 10–12. Confluent and G_0/G_1 -synchronized cell cultures responded with a single major peak of transglutaminase activity at 7–10 h, whereas 50% confluent cultures exhibited a more fluctuating pattern, as observed by others (Scott & Russell, 1982; Korner & Bachrach, 1985, 1987). It can also be seen in Fig. 2 that upon refeeding the G_0/G_1 -synchronized cell cultures, the transglutaminase activity decreased in the first hours after refeeding. This phenomenon was further investigated using the protein synthesis inhibitor cycloheximide during the serum stimulation of the cell cultures. The results of these experiments are shown in Fig. 3 and demonstrate that transglutaminase activity in G_0/G_1 -synchronized confluent cultures decreases rapidly with a half-life of 128 min, whereas no variation in transglutaminase activity was observed in confluent non-synchronized cell cultures under similar conditions.

Fig. 3. Determination of the half-life of endothelial cell transglutaminase in G_0/G_1 -synchronized (Δ) and non-synchronized ($\square$) confluent cultures

The cell cultures were stimulated by replacing the old medium with fresh medium containing 10% FCS supplemented with 4 μ g of cycloheximide/ml. Transglutaminase activity was determined at the indicated times; the zero time was 10 min after medium replacement ($n = 4$). The line for the transglutaminase activity in the synchronized cells represents the best-fitted linear regression equation.

Intracellular distribution of transglutaminase activity at different proliferative states

The intracellular distribution of transglutaminase activity was investigated in cell lysates obtained from cells at the same three different states of proliferation studied

above. Enzyme activity was determined in the cell lysate, and in the 15000 g cytosolic and particulate fractions of cells of passages 6–8 (Table 1). Specific transglutaminase activity was lowest in the proliferating 50% confluent cells and highest in the 100% confluent G_0/G_1 -synchronized cells. In fact, the specific activity in the cell lysate of the latter cells was 1.7-fold higher than that in the 100% confluent non-synchronized cells. Similar ratios were observed when the 15000 g cytosolic and particulate fractions of these different 100% confluent cells were compared. The specific transglutaminase activities of the soluble fractions of both of the 100% confluent cells were about 2.5 times as high as those of the original cell lysate activities. Both of the 100% confluent cells had transglutaminase activities in the 15000 g soluble fractions that were about 40% higher than those in the original unfractionated cell lysates (Table 1, last column). This phenomenon, however, was not observed in the 50% confluent cultures. Thus, the recoveries of the transglutaminase activities in the original cell lysates differed significantly depending on the state of cell proliferation, i.e. being about 90% in the 50% confluent cells, and about 165% and 155% in the 100% confluent non-synchronized and synchronized cells respectively. The particulate fractions of the 100% confluent cells contained only approx. 10–20% of the original cell lysate transglutaminase activity. This was higher than the relative transglutaminase activity of the particulate fraction of the 50% confluent cells, which was only 4% of the original cell lysate activity.

Immunoblotting of transglutaminase from cells at different proliferative states

Fig. 4 shows an autoradiogram of a Western blot after separation on SDS/PAGE of aliquots from the 15000 g

Table 1. Intracellular distribution of transglutaminase activities in endothelial cell cultures at different proliferative states

The cell cultures were grown to the predetermined degrees of confluence and harvested as described in the Experimental section. After the cell extracts had been prepared, aliquots of the cell lysates (0.5–0.6 ml) were centrifuged at 15000 g for 5 min at 4 °C. The supernatants and the washed pellets were collected. The pellets were resuspended in TED buffer (0.25–0.3 ml) and sonicated for 15 s. Aliquots of 50 and 100 μ l of each cellular fraction were assayed for transglutaminase activity and protein concentration. Reported values are means of duplicates from two separate experiments

Cell preparation	Volume (ml)	Protein (mg) (%)	Specific activity*	Transglutaminase (units)† (% of total)
Proliferating cells (50% confluent cultures):				
Cell lysate	0.6	0.560 (100)	1.29	0.723 (100)
Supernatant	0.6	0.288 (51.4)	2.18	0.630 (87.1)
Pellet	0.25	0.273 (48.7)	0.098	0.030 (4.1)
Recoveries		0.561 (100.1)		0.660 (91.2)
100% confluent, non-synchronized cultures:				
Cell lysate	0.5	2.14 (100)	2.75	5.90 (100)
Supernatant	0.5	1.10 (51.4)	7.63	8.40 (142.3)
Pellet	0.3	0.81 (37.8)	2.86	1.32 (22.4)
Recoveries		1.91 (89.2)		9.72 (164.7)
100% confluent, G_0/G_1 -synchronized cultures:				
Cell lysate	0.5	1.37 (100)	4.65	6.38 (100)
Supernatant	0.5	0.80 (58.4)	11.50	9.17 (143.7)
Pellet	0.25	0.40 (29.2)	5.80	0.737 (11.6)
Recoveries		1.20 (87.6)		9.91 (155.3)

* Specific activity in nmol of putrescine/min per mg of protein was derived by dividing the observed transglutaminase activity (units) by the relevant protein concentration.

† One transglutaminase unit is defined as the enzymic activity capable of incorporating 1 nmol of putrescine into casein per min.

Fig. 4. Immunoblot of transglutaminase from endothelial cell cultures at different proliferative states

Protein aliquots (50 μ g) of 15000 *g* cytosolic and particulate fractions prepared from cell lysates were subjected to SDS/PAGE. The transglutaminase antigen was revealed by Western blot techniques, as described in the Experimental section. Lane 1, purified rat liver transglutaminase; lanes 2–4, supernatants of 50% confluent cells, 100% confluent non-synchronized cells and 100% confluent G_0/G_1 -synchronized cells respectively; lanes 5–7, pellets of 50% confluent cells, 100% confluent non-synchronized cells and 100% confluent G_0/G_1 -synchronized cells respectively. The scanning densitometry values for the individual bands were normalized for that of purified rat liver transglutaminase. These were 1.00, 0.50, 1.44, 1.56, non-detectable, 0.81 and 1.28, for lanes 1 to 7.

fractions shown in Table 1. These data illustrate that the transglutaminase antigen of the cytosolic fractions of confluent and G_0/G_1 -synchronized cells is greater than the cytosolic antigen of semi-confluent cells. Most striking is the finding that no transglutaminase band was observed in the particulate fraction of the semi-confluent cells; only when the autoradiograms were over-exposed could a faint band be discerned. The amounts of the transglutaminase antigen of the particulate fractions of confluent and G_0/G_1 -synchronized cells are greater than those indicated by the assayed activities listed in Table 1, which only showed about 10–20% of total cell lysate activities. The pellet/supernatant ratio of transglutaminase activity was 0.16 and 0.08 in the confluent non-synchronized and G_0/G_1 -synchronized cells respectively, whereas these ratios were 0.56 and 0.82 respectively based on densitometric scanning of the bands in Fig. 4. Since this apparent discrepancy in assayed and immunodetected antigen could possibly result from different rates of proteolysis of transglutaminase in cells at various states of proliferation, these experiments were repeated where one of three different protease inhibitors (aprotinin, soybean trypsin inhibitor or PMSF) was added during the harvesting of the cell cultures. No significant differences were observed in the transglutaminase antigen under these conditions. The apparent molecular mass of soluble and particulate transglutaminase as evaluated by SDS/PAGE was approx. 80 kDa, and no lower molecular mass bands were observed. These results, therefore, indicate that the 15000 *g* particulate fraction contains considerable amounts of transglutaminase, not all of which is active under the conventional assay conditions, but which is cross-reactive with the antibody

raised against rat liver soluble transglutaminase. Furthermore, these data suggest that the increase in transglutaminase antigen in confluent cell cultures is greater than the increase in its enzyme activity. One possible explanation for these apparent discrepancies is the existence of a cellular inhibitor of transglutaminase. This hypothesis was addressed in a series of experiments involving the use of dissociative reagents and mixing experiments involving the cytosolic and particulate fractions.

Evidence of a transglutaminase inhibitor in the particulate fraction

The possibility of the existence of a transglutaminase inhibitor was first addressed in an experiment using cell lysates that had been depleted of assayable transglutaminase by means of immunoprecipitation and purified rat liver transglutaminase. When the transglutaminase-depleted cell lysate was added to the purified transglutaminase, dose-dependent inhibition of measured transglutaminase activity was observed (Fig. 5). This

Fig. 5. Inhibition of purified rat liver transglutaminase activity by transglutaminase-depleted endothelial cell lysates

Cell lysates were first depleted of transglutaminase. This involved dissolution in 0.1% Nonidet P-40/0.5 M-NaCl in TED, buffer and incubation with 100 μ g of IgG fraction of the transglutaminase antiserum for 16 h at 4 $^{\circ}$ C. Then 100 μ g of 10% (w/v) Protein A-Sepharose, which had been washed and resuspended in 0.15 M-NaCl in TED buffer, was added and the immunocomplexes precipitated by centrifugation at 250 *g* for 5 min. The supernatant was collected and dialysed for 3 h against 1000 vol. of 50 mM-NaCl in TED buffer. These treated cell lysates had no detectable transglutaminase activity. Aliquots were then incubated for 10 min at 4 $^{\circ}$ C with (■) or without (△) 2 μ g of purified rat liver transglutaminase. At the end of the incubation period, equal volumes of the assay reaction mixture were added and transglutaminase activity determined. Each data point represents the average of duplicate determinations.

Fig. 6. Effects of treatments with dissociative agents and detergents on measured transglutaminase activity in 15000 *g* cytosolic (a) and particulate (b) fractions

These fractions were prepared in TED buffer with 50 mM-NaCl, as described in the Experimental section. Duplicate aliquots (100 μ g of protein) were prepared with the different agents, as follows: 1, control; 2, 25 units of thrombin; 3, 0.5 M-NaCl; 4, 0.5% Nonidet P-40; 5, 0.1% SDS; 6, 25 mM-DTT; 7, 75 mM-KSCN and 8, 25 mM-DTT plus 75 mM-KSCN. All incubations were at 4 $^{\circ}$ C for 1 h, except for thrombin, which was at 37 $^{\circ}$ C for 20 min (using its own control). At the end of the incubation period, samples of the pellet fractions were centrifuged at 15000 *g* for 5 min and duplicate aliquots of the supernatant (a) and of the new supernatant (b) were assayed for transglutaminase activity. The transglutaminase assay was conducted as described in the Experimental section except the final concentration of DTT in the assay mixture was reduced to 4 mM. The activity of the control was designated 100% of transglutaminase activity. Results are expressed as means $\pm$ S.D. ($n = 4$).

depleted lysate had no assayable transglutaminase activity under the usual assay conditions, involving a buffer containing 50 mM-NaCl. However, inhibition of transglutaminase by this lysate did not occur when the ionic strength of the buffer was increased to 0.8 M-NaCl, suggesting that the high ionic strength prevented an interaction between transglutaminase and an inhibitor.

Since the 15000 *g* cytosolic fraction of confluent cells had higher transglutaminase activity than the original whole cell lysate (Table 1), it was hypothesized that this might be due to a reversible interaction between transglutaminase and an inhibitor. Preliminary experiments on cell lysates revealed that treatment with 0.5 and 0.8 M-NaCl caused a 35–50% increase in transglutaminase

activity; no increases were observed after similar treatment of purified rat liver transglutaminase. This suggested that some fraction of transglutaminase is bound to a cellular component via an ionic interaction rendering it inactive. In order to examine this hypothesis further, the experiment described in Fig. 6 was conducted, which shows the effects of treatments with different dissociative agents and detergents on transglutaminase activity in the 15000 *g* fractions. These experiments were carried out in the cytosolic and particulate fractions prepared from 100% confluent G₀/G₁-synchronized cells to determine whether transglutaminase activity could be recovered by one or more of these treatments. The synchronized cells were used since their pellet fraction contained only 11% of the total cell lysate transglutaminase activity (Table 1). Treatment of the 15000 *g* particulate fraction with 0.5 M-NaCl and 25 mM-DTT plus 75 mM-KSCN resulted in major increases in measured transglutaminase activity, i.e. 4- and 5-fold increases respectively. Neither thrombin nor neutral or anionic detergents caused increases in particulate transglutaminase activity. To confirm that these effects were not due to an assay artifact or due to specific properties of endothelial cell transglutaminase, similar experiments were conducted on the 15000 *g* supernatant. Although some increases were observed after 25 mM-DTT, 75 mM-KSCN and Nonidet P-40, these effects were minor compared with those observed in the particulate fraction, i.e. only 75% increase after DTT, 50% increase after KSCN, and 30% increase after Nonidet P-40. Thus, these results provided evidence of a dissociable complex between an inhibitory component in the 15000 *g* particulate fraction and transglutaminase.

Experiments were then carried out to determine whether the transglutaminase inhibitor would be in the 100000 *g* particulate fraction, whether it would also inhibit purified rat liver transglutaminase, and whether its inhibitory effect would be dose-dependent. Fig. 7 shows the results of these experiments, illustrating that the activity of both endothelial cell lysate transglutaminase and purified rat liver transglutaminase were inhibited by the 100000 *g* particulate fraction in a dose-dependent manner. Enzyme kinetics experiments were carried out to elucidate the type of inhibition of transglutaminase activity caused by the putative endothelial cell transglutaminase inhibitor. This involved the addition of 5 μ g of the 100000 *g* particulate fraction to purified rat liver transglutaminase, using the same conditions as those described in Fig. 7 for rat liver transglutaminase and different amounts of substrate. The results showed an unchanged K_m and a 3.4-fold decrease in V_{max} (92 versus 27 nmol/min per mg). Control experiments where albumin was added in increasing amounts (0 to 30 μ g) instead of the 100000 *g* particulate fraction did not reveal any changes in enzyme activity, demonstrating that simply the addition of extra protein under these conditions does not cause lowering in measured enzyme activity.

DISCUSSION

Transglutaminases have been described in a variety of tissues and body fluids (Pisano *et al.*, 1968; Folk & Finleyson, 1977; Lorand & Conrad, 1984), and most recently in bovine vascular endothelial cells (Greenberg *et al.*, 1987; Kojima *et al.*, 1987). The studies by Greenberg *et al.* (1987) reported that bovine aortic

Fig. 7. Inhibition of enzymic activity of purified rat liver transglutaminase (□) and 100000 g supernatant endothelial cell transglutaminase (■) by the 100000 g particulate fraction

Increasing amounts of protein of the 100000 g particulate fraction were added to 50 µl of purified rat liver transglutaminase (14.5 transglutaminase units) and 50 µl of the 100000 g cytosolic fraction (9.5 transglutaminase units). Control experiments with added albumin revealed no decrease in enzyme activity. Transglutaminase activity was assayed in duplicate aliquots as described in the Experimental section after incubation at 4 °C for 1 h, and adjustment of buffer volume to 200 µl with TED buffer. Results are expressed as means ± S.D. ($n = 4$).

endothelial cell transglutaminase was $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent and had a molecular mass of 79 kDa as evaluated by SDS/PAGE. Our molecular mass estimate by gel filtration of 88 kDa, compared with 80 kDa for purified rat liver transglutaminase, is slightly higher than the estimate by Greenberg *et al.* (1987) and much higher than that of 58 kDa by Kojima *et al.* (1987), but in good agreement with the estimate for guinea pig liver transglutaminase reported by Connellan *et al.* (1971). The endothelial cell transglutaminase was inhibited by three known inhibitors of tissue-type transglutaminases (Folk & Chung, 1973; Folk & Finleyson, 1977), and the enzyme reaction constants K_m and V_{max} observed are similar to those reported for other tissue-type transglutaminases (Folk & Chung, 1973; Folk & Finleyson, 1977). The transglutaminase activity was found to be markedly dependent on the proliferative state of the endothelial cells. The 100 % confluent cells had higher activity than the 50 % confluent cells, and the 100 % confluent G_0/G_1 growth-arrested cells had still higher activity (Fig. 2). Such an inverse relationship between cellular transglutaminase activity and the proliferative state of cells has been reported for both non-transformed and transformed mammalian cells. This has led to the suggestion by Birckbichler and other investigators for a role of transglutaminase in the regulation of cell proliferation (Birckbichler *et al.*, 1977; Birckbichler & Patterson, 1978; Milhaud *et al.*, 1980; Korner & Bachrach, 1985).

The majority of endothelial cell transglutaminase activity was found in the 15000 g cytosolic fraction, with only a small part of the total cellular activity in the particulate fraction (Table 1). The activity of the par-

ticulate fraction varied from as little as 4.5 % of the original activity in the 50 % confluent cells to as high as 22.4 % in the 100 % confluent non-synchronized cells. These findings are in general agreement with those made in other cell types (Korner & Bachrach, 1985; Slife *et al.*, 1985; Byrd & Lichti, 1987; Barnes *et al.*, 1985). Of particular interest is the observation that the cytosolic fraction of the 100 % confluent cells contained 55–65 % higher transglutaminase activity than the original cell lysate. Investigations provided evidence that endothelial cell transglutaminase is present, at least in part, in a non-active, latent form, that can be 'activated'. First, comparisons of transglutaminase activity and antigen in the 15000 g fractions of non-confluent and confluent cultures revealed a significant discrepancy between these two enzyme parameters. The particulate fraction contained greater amounts of the enzyme (Fig. 4) than was indicated by the measured activity (Table 1), particularly in the confluent cells. These observations imply that in spite of the increased transglutaminase activity in confluent, non-proliferating cells, measurement of its activity provides an underestimation of the actual transglutaminase present. Second, when confluent G_0/G_1 -synchronized cell cultures, which have the highest transglutaminase activity, were released from synchronization by serum feeding in the presence of cycloheximide, a rapid decrease in transglutaminase activity was observed, with a half-life of about 2 h (Fig. 3). This suggests that transglutaminase activity can be rapidly down-regulated without new protein synthesis.

Additional experiments aimed at elucidating the mechanism underlying such down-regulation provided evidence that the regulation of endothelial cell transglutaminase activity involved some type of inhibitory complex formation with a cellular inhibitor. First, the addition of transglutaminase-depleted cell lysate to purified rat liver transglutaminase resulted in a dose-dependent inhibition of transglutaminase activity (Fig. 5). Significantly, such inhibition was abolished in the presence of 0.8 M-NaCl, confirming the hypothesis of a down-regulation of transglutaminase activity by an ionic inhibitory complex formation. Second, treatment of cell lysates from confluent cultures with high salt concentration revealed an increase in transglutaminase activity. This suggested that endothelial cell transglutaminase is in part maintained in an inhibited form by an ionic interaction. Treatment of the 15000 g particulate fraction with 0.5–0.8 M-NaCl and 75 mM-KSCN, and with 20 mM-DTT, resulted in significant increases in transglutaminase activity (Fig. 6), suggesting that the inhibitory complex was primarily in the particulate fraction. This inhibitor was located in the 100000 g particulate fraction, and it inhibited both endothelial cell transglutaminase and purified rat liver transglutaminase (Fig. 7), in an apparently non-competitive manner. The identity and physiological significance of such an inhibitor remains to be determined.

Transglutaminases have been found by several investigators to be associated with cell membranes or cellular matrices. In human red blood cells (Lorand *et al.*, 1986), rat liver (Chang & Chung, 1986; Juprelle-Soret *et al.*, 1988; Slife *et al.*, 1985, 1987), lung cells and matrices (Cocuzzi & Chung, 1986; Juprelle-Soret *et al.*, 1988), chondrosarcoma cells (Chang & Chung, 1986) and human lung fibroblasts (Birckbichler & Patterson, 1978), considerable transglutaminase activity or antigen has

been shown to be associated with detergent-insoluble material. It has been suggested that the plasma membrane transglutaminase may be associated with a distinct macromolecular plasma membrane domain (Tyrrell *et al.*, 1986). It has been suggested that the bound enzyme may play a role in the processes of organ development and tissue regeneration by the formation of covalently cross-linked protein matrices at the sites of cell to cell contacts (Cocuzzi & Chung, 1986; Slife *et al.*, 1987). The exact role of the soluble intracellular enzyme, however, is still unclear, although several cellular functions have been found to correlate with increases in cellular transglutaminase activity (Davies *et al.*, 1980; Fesus *et al.*, 1981; Moore *et al.*, 1984). It has recently been reported that the cytosolic and particulate transglutaminases represent two different enzymes, based on their different chromatographic properties (Chang & Chung, 1986). However, the relationship between these two enzymes remains unclear. It has been proposed that they may indeed represent the same enzyme, perhaps in different conformational forms, that partitions between the cytosol and specific membrane domains in response to various physiological demands (Korner & Bachrach, 1985, 1987; Slife *et al.*, 1987).

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