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Subcellular localization of transglutaminase

Effect of collagen

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1. The subcellular distribution of transglutaminase was investigated by using the analytical approach of differential and isopycnic centrifugation as applied to three organs of the rat: liver, kidney and lung. After differential centrifugation by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans [(1955) Biochem. J. 63, 604–617], transglutaminase is mostly recovered in the unsedimentable fraction S and the nuclear fraction N. After isopycnic centrifugation of the N fraction in a sucrose density gradient, a high proportion of the enzyme remains at the top of the gradient; a second but minor peak of activity is present in high-density regions, where a small proportion of 5′-nucleotidase, a plasma-membrane marker, is present together with a large proportion of collagen recovered in that fraction. 2. Fractions where a peak of transglutaminase was apparent in the sucrose gradient were examined by electron microscopy. The main components are large membrane sheets with extracellular matrix and free collagen fibres. 3. As these results seem to indicate that some correlation exists between particulate transglutaminase distribution and those of collagen and plasma membranes, the possible binding of transglutaminase by collagen (type I) and by purified rat liver plasma membrane was investigated. 4. The binding studies indicated that collagen is able to bind transglutaminase and to make complexes with plasma-membrane fragments whose density is higher than that of plasma-membrane fragments alone. Transglutaminase cannot be removed from such complexes by 1 % Triton X-100, but can be to a relatively large extent by 0.5 M-KCl and by 50 % (w/v) glycerol. 5. Such results suggest that the apparent association of transglutaminase with plasma membrane originates from binding *in vitro* of the cytosolic enzyme to plasma membrane bound to collagen, which takes place during homogenization of the tissue, when the soluble enzyme and extracellular components are brought together.

INTRODUCTION

Several fractionation studies have shown that transglutaminase is mostly recovered in the soluble fraction of the homogenate, but that a significant proportion of the enzyme is always present in sedimentable components (Birckbichler *et al.*, 1976; Griffin *et al.*, 1978; Juprelle-Soret *et al.*, 1984; Slife *et al.*, 1985). Until now, there is no clear information about the nature of the subcellular structures with which the particulate enzyme is associcated. Investigations on rat liver suggest that nearly all the particulate transglutaminase is located in a specific plasma-membrane domain (Slife *et al.*, 1985, 1986). However, these results do not allow one to exclude unequivocally the possibility that particulate transglutaminase could originate from binding *in vitro* of a certain amount of enzyme to the membranes, taking into account that a large proportion of transglutaminase is recovered in the homogenate soluble fraction. Obviously, from a functional point of view it is important to know whether particulate transglutaminase results from an association of the enzyme with a membrane inside the cell or is caused by an artifactual binding. In the work presented here, we have re-investigated the subcellular distribution of transglutaminase, using the analytical approach of differential and isopycnic centrifugation as applied to three organs of the rat: liver, kidney and lung. As our results seemed to indicate that some correlation exists between particulate transglutaminase distribution and that of collagen, we investigated the influence of collagen on transglutaminase sedimentability. Our observations strongly suggest that particulate transglutaminase results from binding the enzyme *in vitro* during homogenization.

EXPERIMENTAL

Tissue fractionation

Experiments were performed with male Wistar rats weighing 200–250 g. Differential centrifugation was performed as described by de Duve *et al.* (1955). A nuclear fraction (N), a heavy-mitochondrial fraction (M), a light-mitochondrial fraction (L), a microsomal fraction (P) and a soluble fraction (S) were isolated.

Density-gradient experiments were carried out as described by Beaufay *et al.* (1964) on N fractions isolated in 0.25 M-sucrose by differential centrifugation and treated in an Ultra-Turrax homogenizer for 2 × 15 s. For details of the centrifugation conditions see the text.

Purified liver plasma-membrane preparations were obtained by the method of Brown *et al.* (1976).

Enzyme assays

Marker enzymes for the main subcellular membranes were assayed by the methods described in the indicated references: acid phosphatase (Appelmans *et al.*, 1955), cytochrome oxidase (Appelmans *et al.*, 1955), glucose-6-phosphatase (de Duve *et al.*, 1955) and 5′-nucleotidase (Wattiaux-De Coninck & Wattiaux, 1969). Trans-

glutaminase was assayed by measuring [^{3}H]putrescine incorporation into dimethylcasein by the method described by Lorand *et al.* (1972) with the modifications described by Juprelle-Soret *et al.* (1984).

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, DNA by that of Kapuscinski & Skoczylas (1977), and collagen by that of Woessner (1961).

Binding experiments

Purified guinea-pig liver transglutaminase (Sigma Chemical Co.) was used, with an activity (per mg of protein) on average 3000 times that found in guinea-pig liver homogenate. Labelling with ^{125}I was performed by the chloramine-T method (Greenwood *et al.*, 1963). Binding was performed by incubating labelled transglutaminase (0.1 μg of protein) in 0.1 ml of a medium containing 10 mM-Tris/HCl buffer, pH 7.5, 20 mM-NaCl, 0.1 mM-$CaCl_2$, 0.4% (w/v) bovine serum albumin and, when required, collagen and plasma membrane as explained in the text. After 1 h at 0 °C, a sample of the preparation was layered at the top of a sucrose gradient whose density ranged from 1.05 to 1.32 g/ml, and centrifuged for 3 h at 65000 rev./min in a Spinco SW 65 rotor.

Morphological examination

For electron-microscopic examinations, samples of fractions isolated by isopycnic centrifugation were kept at 0 °C in the presence of 1% (w/v) glutaraldehyde and 0.1 M-cacodylate buffer, pH 7.4, after which the preparations were centrifuged for 15 min in a bench centrifuge and the pellets were processed as described by Wattiaux *et al.* (1978).

Materials

Horse heart cytochrome *c*, glucose 6-phosphate (sodium salt), 5'-AMP, guinea-pig liver transglutaminase and calf skin collagen (type I) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Sodium glycerophosphate was from Merck (Darmstadt, Germany), and [^{3}H]putrescine from NEN Chemicals (Dreieich, Germany).

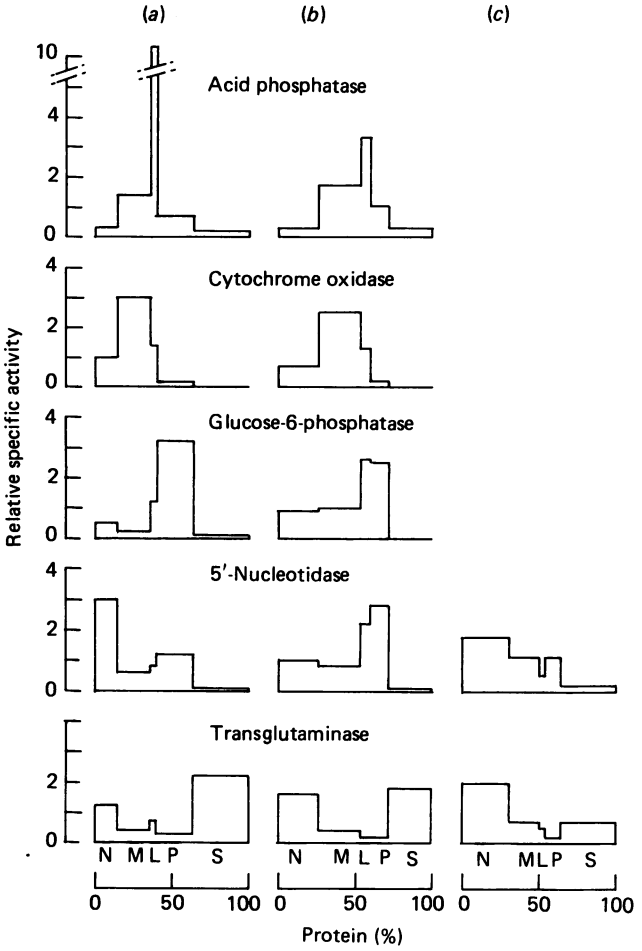

Fig. 1. Distribution patterns of transglutaminase and reference enzymes after differential centrifugation by the procedure of de Duve *et al.* (1955)

The ordinate shows relative specific activity (percentage of total recovered activity/percentage of total recovered protein), and the abscissa relative protein content of fractions (cumulatively from left to right): N, nuclear fraction; M, heavy-mitochondrial fraction; L, light-mitochondrial fraction; P, microsomal fraction; S, soluble fraction. (*a*) Liver; (*b*) kidney; (*c*) lung.

RESULTS

Differential centrifugation

Fig. 1 illustrates the distribution of transglutaminase and of several reference enzymes after differential centrifugation (de Duve *et al.*, 1955). In agreement with previous observations, transglutaminase is mostly recovered in the unsedimentable fraction S and in the nuclear fraction N. However, important differences are observed between organs, the largest proportion of sedimentable enzyme being recovered in lung homogenates.

Clearly, transglutaminase distribution does not correspond to that of any of the reference enzymes. There is one marker enzyme that, like transglutaminase, is recovered to a relatively large extent and exhibits a peak of relative specific activity in N fraction: this is 5'-nucleotidase, a plasma-membrane marker. However, this enzyme is also largely recovered in the microsomal fraction P, which is almost devoid of transglutaminase.

Besides cytoplasmic structures, the N fraction contains most of the cell nuclei (90% of the homogenate DNA) and extracellular-matrix components; we found that 35% (liver), 60% (kidney) and 75% (lung) of homogenate collagen is recovered in this fraction.

Isopycnic centrifugation

The nuclear fraction N was analysed by isopycnic centrifugation in a sucrose density gradient. As shown in Fig. 2, a similar behaviour is observed for transglutaminase in the three organs. A high proportion of the enzyme remains at the top of the gradient, mainly for liver and lung, indicating that a large amount of the enzyme has been released from the structures with which it was associated. A second peak of activity is present in high-density regions of the gradient. The distribution of the plasma-membrane enzyme 5'-nucleotidase is bimodal: a large proportion of the enzyme is found in relatively light-density regions of the gradient, but no transglutaminase is recovered in these regions. 5'-Nucleotidase exhibits a second peak of activity,

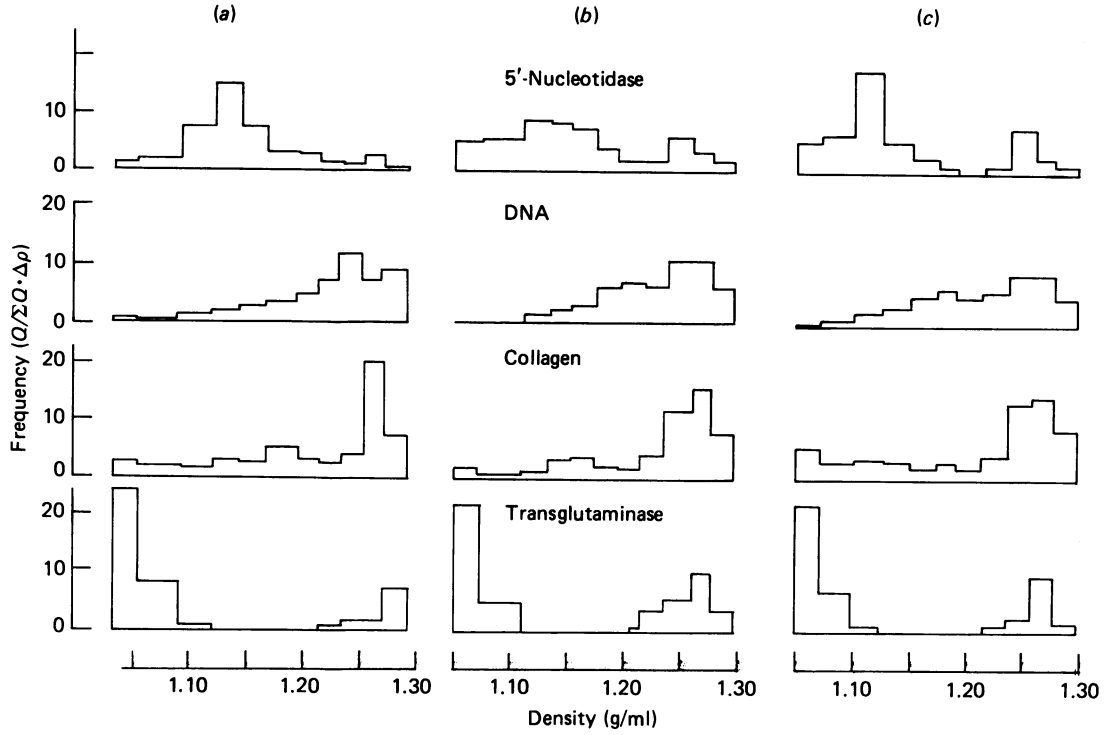

Fig. 2. Density distribution histograms of transglutaminase, collagen, DNA and 5'-nucleotidase after isopycnic centrifugation of an N fraction

Centrifugations were performed at 39000 rev./min in a Spinco SW 65 rotor. The time integral of the square angular velocity was 144 rad^2/ns. The sucrose gradient extended from 1.09 to 1.32 g/ml density. Ordinate: frequency $Q/\Sigma Q \cdot \Delta\rho$, where Q represents the activity found in the fraction, ΣQ the total recovered activity and $\Delta\rho$ the increment of density from top to bottom of the fraction. (*a*) Liver; (*b*) kidney; (*c*) lung.

markedly less pronounced, in the high-density zones where sedimentable transglutaminase is located. However, there is no strict coincidence between transglutaminase distribution and that of 5'-nucleotidase found in these gradient zones. The distributions of acid phosphatase, cytochrome oxidase and glucose-6-phosphatase were also measured; they were quite different from that of transglutaminase and are not reported in Fig. 2.

Taking into account that N fraction contained most of the DNA and a large proportion of collagen, we have established the distribution of these components in the same gradients. DNA exhibits a relatively flattened distribution, which probably results from disruption of some nuclei during homogenization and centrifugation. The DNA distributions are distinctly different from that of transglutaminase. Collagen is recovered in all the gradient fractions, but is more abundant in high-density zones, where sedimentable transglutaminase equilibrates.

Fractions where a peak of particulate transglutaminase was apparent in the sucrose gradient were examined under the electron microscope. As shown by Fig. 3, the main components are large membrane sheets with extracellular matrix and free collagen fibres.

Binding of transglutaminase *in vitro*

Results reported by Saito *et al.* (1986) indicate that Factor XIII, a zymogen form of transglutaminase, could be bound to collagen. As our centrifugation results show that after centrifugation particulate transglutaminase is recovered in zones where most of the collagen present in fraction N is located, it may be asked whether the enzyme does not bind to collagen fibres or to collagen linked to plasma-membrane fragments during homogenization of the liver. We performed binding experiments *in vitro* with purified guinea-pig liver transglutaminase labelled with ^{125}I. Unexpected difficulties were met in trying to obtain a sufficiently pure rat liver transglutaminase. Therefore we decided to utilize the more frequently used guinea-pig liver enzyme. We found that an N fraction is able to bind *in vitro* a certain amount of the radioactive compound. In addition, when fractions isolated by isopycnic centrifugations of fraction N are incubated in the presence of labelled transglutaminase, the maximum binding is observed in the gradient regions where endogenous transglutaminase is located, as exemplified by results reported in Fig. 4.

In a second set of experiments, we looked for the possible binding of transglutaminase by collagen, by purified rat liver plasma membrane and by collagen mixed with purified plasma membrane. We used collagen type I (from calf skin) because it represents, with collagen type III (which is structurally similar), a large part of the total liver collagen (Rojkind & Perez-Tamayo, 1983). The binding was assessed by centrifuging the mixture in a sucrose gradient and by determining the radioactivity of the different fractions recovered after centrifugation, and, when required, their contents of 5'-nucleotidase and collagen. Fig. 5 shows the distribution of radioactivity after centrifuging transglutaminase alone. Almost all the

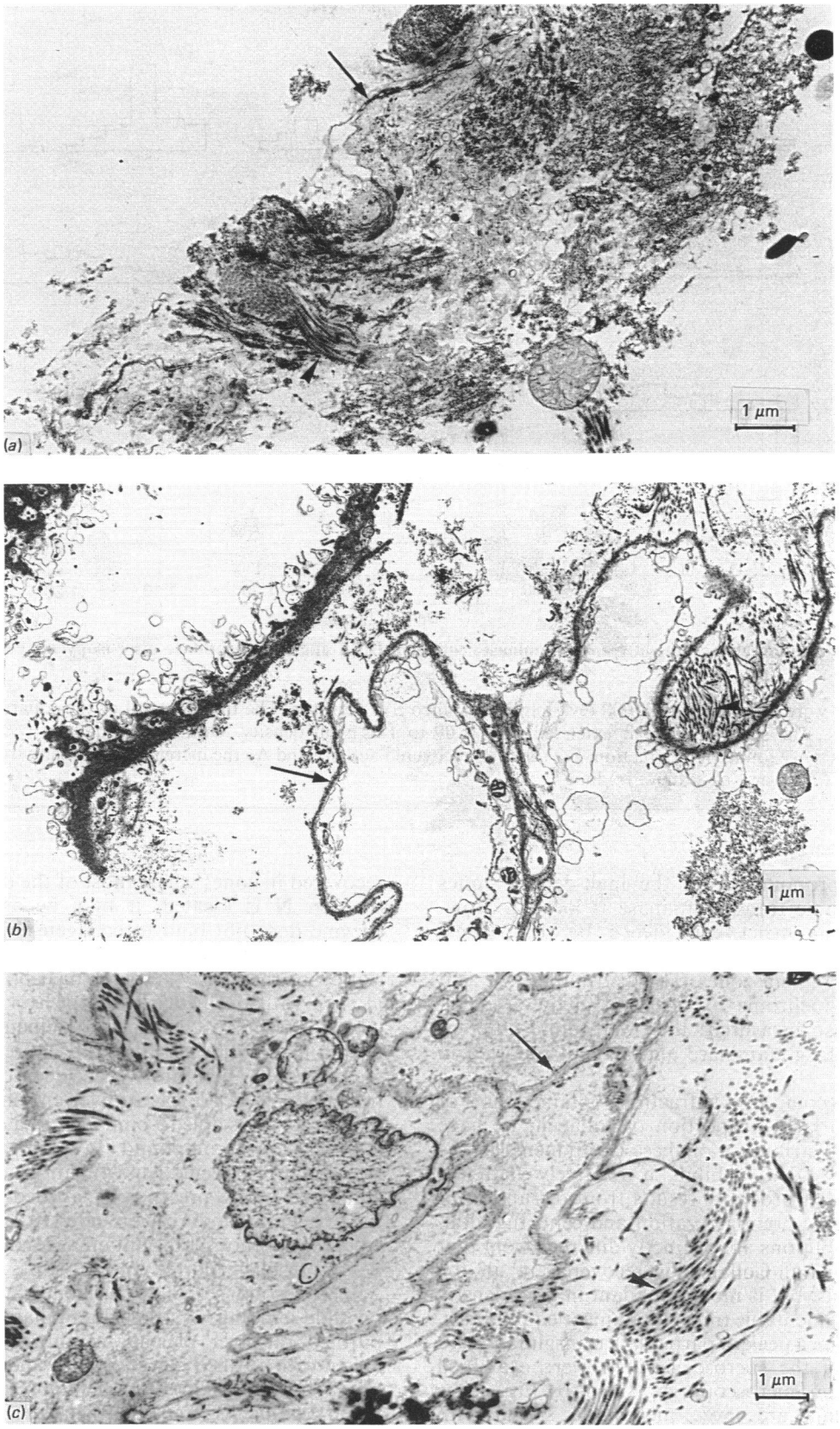

Fig. 3. Morphological aspect of gradient fractions

Centrifugation of N fractions was done in a sucrose gradient as described in Fig. 2. Electron-microscopic examination was performed on fractions recovered in the dense regions of the gradient, exhibiting a peak of transglutaminase activity. (*a*) Liver; (*b*) kidney; (*c*) lung. Note the presence of large membrane sheets associated with extracellular matrix (→) and numerous collagen fibres (➤).

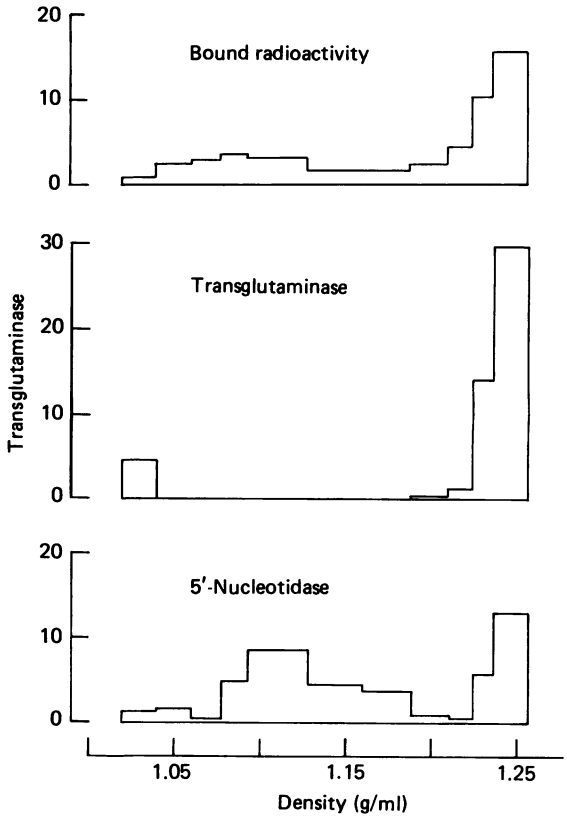

Fig. 4. Density distribution histograms of bound radioactivity, transglutaminase and 5'-nucleotidase

An N fraction was isolated from a kidney homogenate and subjected to isopycnic centrifugation as described in the legend of Fig. 2, except that the upper density limit of the gradient was 1.26 g/ml. Samples of the fractions recovered after centrifugation were incubated in presence of ^{125}I-transglutaminase (0.1 μg of protein) for 1 h at 0 °C in a medium containing 10 mM-Tris/HCl buffer, pH 7.5, 20 mM-NaCl, 0.1 mM-$CaCl_2$ and 0.4% (w/v) bovine serum albumin. After that, the tubes were centrifuged at 39000 rev./min for 1 h in a Spinco 40 rotor, and the radioactivity of the pellets was measured. Endogenous transglutaminase and 5'-nucleotidase activities were determined on samples of the same fractions.

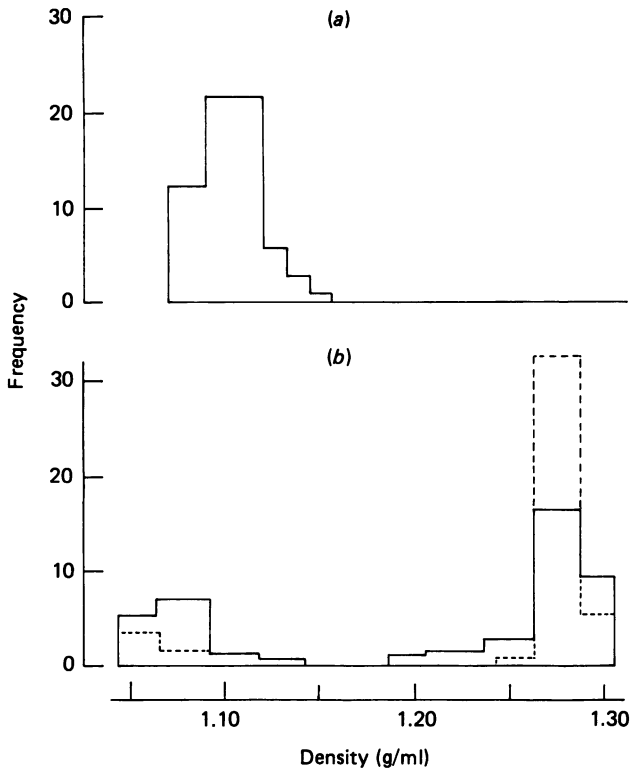

Fig. 5. Density distribution histograms of ^{125}I-transglutaminase

^{125}I-transglutaminase (0.1 μg of protein), alone (*a*) or mixed with 100 μg of collagen (*b*), was incubated for 1 h at 0 °C in the medium described in Fig. 4 legend. After that, the preparations were layered at the top of a sucrose gradient extending from 1.05 to 1.32 g/ml density and centrifuged for 3 h at 65000 rev./min (480 rad^2/ns) in a Spinco SW 65 rotor. Fractions were collected and analysed for radioactivity (——) and in (*b*) for their collagen content (– – –).

radioactivity remains at the top of the gradient. Fig. 5 also shows the radioactivity distribution when the enzyme has been mixed with collagen (100 μg). Under these conditions a large proportion of radioactivity migrates in the gradient and is recovered near the bottom of the tube, where most of the collagen is situated. The distribution of radioactivity when labelled transglutaminase was mixed with purified plasma membrane (100 μg of protein) is illustrated in Fig. 6. Only a small peak of radioactivity can be seen where plasma membrane equilibrates, as ascertained by 5'-nucleotidase distribution. Fig. 6 also shows the distribution of radioactivity when labelled transglutaminase is kept in the presence of collagen (100 μg) and plasma membrane (100 μg of protein) together. Contrarily to what is observed with collagen alone, no radioactivity is recovered at the bottom of the gradient. A peak of radioactivity is now located where the plasma-membrane fragments equilibrate, as ascertained by the distribution of 5'-nucleotidase. That peak is markedly more pronounced than when incubation took place in the absence of collagen. The distribution of collagen shows that a large proportion of the compound is now found where 5'-nucleotidase is recovered. Another point is that the distribution of this enzyme is shifted towards higher density: the median equilibrium density is 1.154 g/ml instead of 1.134 g/ml. Such a result strongly suggests that a certain amount of collagen to which transglutaminase is linked has been bound to plasma-membrane fragments, causing an increase in their density. Results presented in Fig. 7 strengthen such a hypothesis. In one experiment plasma membrane (100 μg of protein) was mixed with collagen (1 μg) and radioactive transglutaminase. In another assay the amount of plasma membrane was decreased (30 μg of protein) and that of collagen was increased (100 μg). It is obvious that the equilibrium density of plasma membrane, as indicated by 5'-nucleotidase distribution, depends on the ratio plasma membrane/collagen. It increases when that ratio decreases. In addition, the proportion of radioactivity that follows 5'-nucleotidase increases when the amount of collagen increases.

It has been shown that transglutaminase present in plasma membrane preparation cannot be released by Triton X-100; a certain proportion is solubilized by washing with salt or after incubation at 37 °C in the

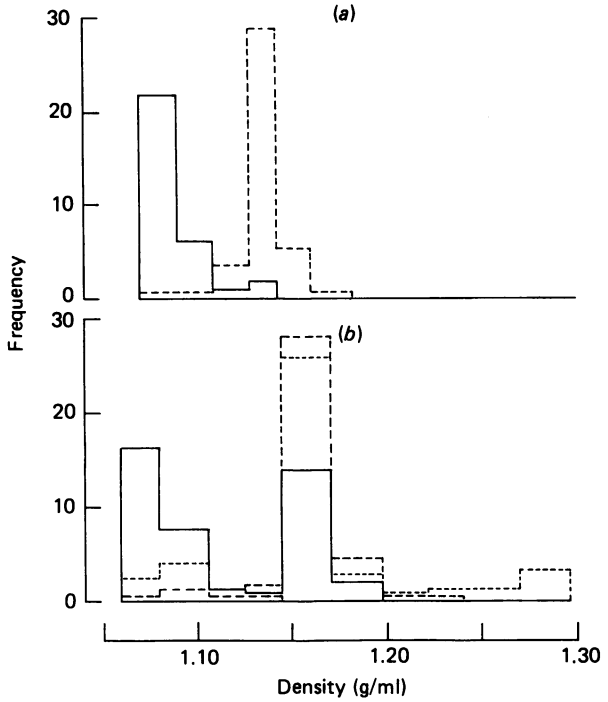

Fig. 6. Density distribution histograms of ^{125}I-transglutaminase and 5′-nucleotidase and collagen

(*a*) Rat liver purified plasma membranes (100 μg of protein) were incubated in presence of ^{125}I-transglutaminase (0.1 μg) for 1 h at 0 °C in the medium described in Fig. 4 legend. After that, the preparation was processed as described in Fig. 5 legend. (*b*) Similar experiment to that in (*a*), except that 100 μg of collagen was present in the incubation medium. ——, Radioactivity; – – – –, 5′-nucleotidase; ------, collagen.

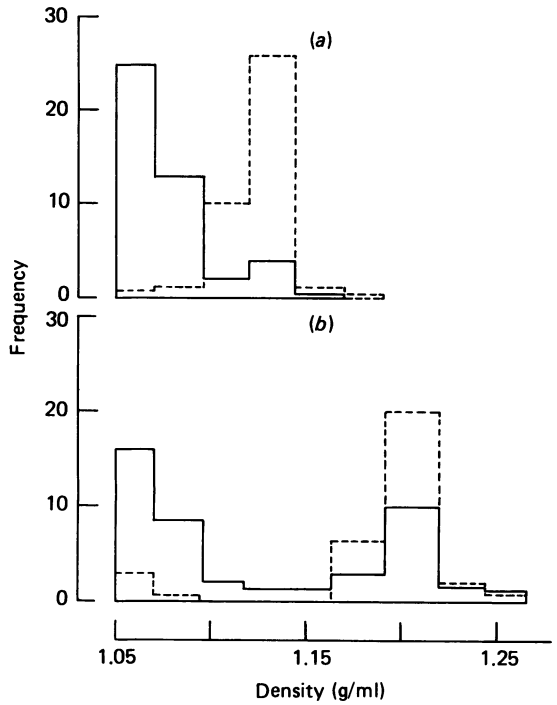

Fig. 7. Density distribution histograms of ^{125}I-transglutaminase and 5′-nucleotidase

(*a*) Rat liver purified plasma membranes (100 μg of protein) and collagen (1 μg of protein) were incubated in presence of ^{125}I-transglutaminase (0.1 μg of protein) for 1 h at 0 °C in the medium described in Fig. 4 legend. After that, the preparation was processed as described in Fig. 5 legend. (*b*) Similar experiment to that in (*a*) except that the amount of plasma membrane was 30 μg of protein and the amount of collagen 100 μg. ——, Radioactivity; – – – –, 5′-nucleotidase.

presence of 50% glycerol (Slife *et al.*, 1985, 1986). Artificial transglutaminase–collagen–plasma membrane complex was isolated by gradient centrifugation and subjected to similar treatments. We found that treatment of such a preparation with 1% (w/v) Triton X-100 released only a small percentage of the radioactivity. Washing with 0.5 mM-$MgCl_2$/0.5 M-KCl releases 45% of the radioactivity; according to Slife *et al.* (1985), the same solution solubilizes 47% of transglutaminase present in purified plasma membranes. Incubation at 37 °C with 50 mM-dithiothreitol does not release labelled transglutaminase from the artificial complex; on the other hand, 50% glycerol causes 75% release. Similar results have been obtained for transglutaminase associated with purified plasma membrane (Slife *et al.*, 1985, 1987).

Binding experiments were performed, as described, in a medium containing several solutes. In fact, we later found that the binding of ^{125}I-transglutaminase to collagen–plasma membrane complex is not affected by the presence of these components, and can take place in a medium containing only 0.25 M-sucrose.

To be sure that the radioactivity bound is really a binding of transglutaminase, the protein's ability to bind to a mixture of collagen and purified plasma membrane was assessed by determining in two parallel experiments the bound enzymic activity and the bound radioactivity in the presence of increasing concentrations of transglutaminase in the medium. The two determinations gave similar results; in both cases the amount of bound transglutaminase linearly increases with the concentration of transglutaminase in the medium. The slope of the straight line indicates a binding of 25% of transglutaminase present in the medium by the enzymic method and of 30% by the radioactive determination.

DISCUSSION

In agreement with previous observations (Birckbichler *et al.*, 1976; Griffin *et al.*, 1978; Juprelle-Soret *et al.*, 1984; Slife *et al.*, 1985), our fractionation experiments show that, in liver, kidney and lung homogenates, a significant proportion of transglutaminase is recovered in a fraction sedimenting at low centrifugation speed, relatively enriched in plasma-membrane fragments. These structures with which particulate transglutaminase is associated exhibit a high equilibrium density in a sucrose gradient. On the other hand, results presented in the second part of this paper illustrate that it is possible to create artificial complexes of transglutaminase–collagen and transglutaminase–collagen–plasma membrane. Is there a relationship between the first and the second set of observations: does binding to membranes of transglutaminase occur *in vitro* during tissue homogenization, and therefore is particulate transglutaminase an artifact? Obviously an objection can be raised to our binding experiments, owing to the fact that the material

involved is heterologous: guinea-pig liver enzyme, calf skin collagen, rat liver plasma membrane. However, with respect to collagen, we think that, if calf skin collagen can attach to rat liver plasma membrane and bind transglutaminase, it is probable that the same physicochemical type of collagen (collagen I) originating from the liver would have the same properties. On the other hand, as mentioned above, difficulties encountered in purification of rat liver transglutaminase hampered our endeavour to make use of that enzyme. To our knowledge there are no data that allow us to compare the structure of tranglutaminase originating from the two animal species. Therefore we have to extrapolate the binding properties that we have described for the guinea-pig transglutaminase to the rat enzyme. It is worthwhile to mention here that another kind of transglutaminase, bovine platelet Factor XIII, is also able to bind to collagen (Saito *et al.*, 1986); this strengthens the hypothesis that the binding of transglutaminase to collagen does not strictly depend on the origin of the enzyme.

If our binding results can be extrapolated to the rat liver transglutaminase, we believe that there are strong indications that particulate transglutaminase is an artifact. Firstly, in the homogenate of the three organs that we investigated, transglutaminase is always found to a large extent in the unsedimentable fraction. In addition, a large proportion of transglutaminase that sediments at low speed in the N fraction is easily removed when that fraction is layered at the top of a sucrose gradient, and is therefore probably soluble enzyme loosely adsorbed on structures present in fraction N. Hence the proportion of 'true' particulate transglutaminase is markedly lower than that which is observed after differential centrifugation. Secondly, localization of transglutaminase in plasma membrane is poorly supported by centrifugation results, as ascertained by the behaviour of the enzyme after differential and isopycnic centrifugation in comparison with that of 5'-nucleotidase, a plasma-membrane marker, the differences in distribution of the two enzymes being more prominent than the similarities. To be present in plasma membrane, transglutaminase would have to be located in limited membrane sites recovered in the homogenate as fragments with a large sedimentation coefficient and a high density. To exhibit such a high density, these fragments have to be associated with extracellular-matrix components that probably contain collagen. Even purified plasma-membrane preparation contains significant amounts of collagen (it represents 2% of the protein in the preparations that we have used). Therefore, from our binding experiments, it seems very probable that, when the cells are disrupted and the soluble enzyme and extracellular components are brought together, cytosolic transglutaminase binds to such plasma-membrane fragments. The binding *in vitro* that we have described takes place at 0 °C and is efficient in 0.25 M-sucrose without any other solutes. Thirdly, endogenous particulate transglutaminase, as well as transglutaminase bound *in vitro*, could not be solubilized by Triton X-100, but to some extent it could be by salts or by glycerol. This suggests that the binding is similar in both cases, and particularly that membrane lipids are not involved.

Obviously one cannot totally exclude the possibility that the association *in vitro* of transglutaminase with collagen–plasma membrane is an illustration of a process that takes place in the cell. But, under these conditions, particulate transglutaminase would have to be an extracellular enzyme or to be located at the extracellular face of the plasma membrane, so as to be in contact with collagen. Such a situation would require a particulate transglutaminase different from the cytosolic enzyme; indeed, its biosynthesis would necessarily require a mechanism involved in plasma-membrane or secretion-protein biosynthesis that is distinct from that used for cytosolic proteins.

It has been proposed that particulate transglutaminase could play a role in clustering receptors for endocytosis (Davies *et al.*, 1980), by forming cross-linked protein matrices in areas of cell-to-cell contact (Slife *et al.*, 1986). Our observations, by suggesting that particulate transglutaminase results from an artifactual binding of the soluble enzyme *in vitro*, casts some doubt on the putative role of the enzyme in membrane function.

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