Subcellular Localization of γ -Glutamyltransferase in Calf Thymocytes

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The subcellular localization of γ -glutamyltransferase in calf thymocytes was investigated and compared with that of alkaline phosphodiesterase I, alkaline nitrophenyl phosphatase, succinate-tetrazolium oxidoreductase (succinate-INT reductase) and lactate dehydrogenase, after two different methods of cell disruption and differential centrifugation. Most of the activity was recovered in the crude membrane fractions (43.0%), but significant amounts co-pelleted with the large-granule (mitochondrial) fractions (31%). The specific activity of the γ -glutamyltransferase in the purified plasma membrane was 30–50 times that of the enzyme in the cell homogenate and had a similar subcellular distribution to the plasma-membrane markers, alkaline phosphodiesterase I and alkaline nitrophenyl phosphatase. It was concluded that γ -glutamyltransferase was primarily a plasma-membrane-bound enzyme, and that its location in other subcellular fractions was probably due to their contamination with plasma-membrane vesicles.

The presence of γ -glutamyltransferase (EC 2.3.2.2), an enzyme concerned in the catabolism of glutathione, has been reported in many mammalian tissues, including kidney, submaxillary glands, prostate, bronchi, seminal vesicles, lactating breast, epididymis, fallopian tubes, brain and intestine (Meister & Tate, 1976).

More recently its presence has been reported in a variety of lymphoid cells, and it has been suggested by Novogrodsky *et al.* (1976) that it is located on the cell surface.

The aim of this work was to investigate more fully this suggestion by following the subcellular localization of γ -glutamyltransferase in calf thymocytes by established methods of cell disruption and plasma-membrane isolation.

Materials and Methods

Preparation of subcellular fractions

Preparation of subcellular components from calf thymocyte suspensions was performed either by the methods of Crumpton & Snary (1974) after cell disruption in a Stansted cell-disrupting pump (Stansted Fluid Power Ltd., Stansted, Essex, U.K.) or after nitrogen cavitation (Ferber *et al.*, 1972; Brunner *et al.*, 1977).

Abbreviations used: ($\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$)-ATPase and ($\text{Na}^+ + \text{K}^+$)-ATPase, ($\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$)-dependent and ($\text{Na}^+ + \text{K}^+$)-dependent adenosine triphosphatase.

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Isolation of plasma membranes after pump disruption

The crude-membrane pellet (19 200g_{av.} for 45 min) was resuspended in 37% (w/v) sucrose dissolved in 10mM-Tris/HCl, pH 7.4. A step gradient was then prepared of equal volumes of 37% sucrose in 10mM-Tris/HCl, pH 7.4, containing suspended membranes and 25% (w/v) sucrose in the same buffer overlaid with 0.25 vol. of 10mM-Tris/HCl, pH 7.4. After centrifugation at 58 000g_{av.} for 18 h (Beckman SW27 rotor operated at 4°C), two plasma-membrane bands, PM₁ and PM₂ with densities of 1.038–1.085 g/cm³ and 1.094–1.132 g/cm³ respectively, and a pellet were obtained. The material in the bands was sedimented at 177 000g_{av.} for 45 min. The sedimented band material and gradient pellet were then washed twice by resuspending in 10mM-Tris/HCl, pH 7.4, and resedimenting, before finally resuspending in the same buffer.

Isolation of plasma membranes after nitrogen cavitation

The method was identical with that described by Brunner *et al.* (1977), except that two large-granule pellets were sedimented after centrifugation of the nuclear-pellet supernatant, at 4800g_{av.} for 10 min (fraction G) and 19 200g_{av.} for 20 min (fraction H), before the crude-membrane fraction was pelleted at 177 000g_{av.} for 60 min.

Biochemical determinations

The activity of alkaline nitrophenyl phosphatase (EC 3.1.3.1) was determined as described previously (Ferber *et al.*, 1976). The activity of γ -glutamyl-

transferase was determined as described by Novogrodsky *et al.* (1976), with L- γ -glutamyl 3-carboxy-4-nitroanilide and glycylglycine (Boehringer, Mannheim, W. Germany) as substrates, but trichloroacetic acid (10%, w/v) was used instead of acetic acid to stop the reaction. Succinate-tetrazolium oxidoreductase [succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase; succinate-INT reductase] was assayed as described by Pennington (1961). Alkaline phosphodiesterase I (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) and 5'-nucleotidase (EC 3.1.3.5) were assayed as described by Evans *et al.* (1973). Cytochrome *P*-450 was assayed as described by Burke & Mayer (1974), glucose 6-phosphatase (EC 3.1.3.9) by the method of Appelmans *et al.* (1955) and ($\text{Mg}^{2+} + \text{Na}^{+} + \text{K}^{+}$)-ATPase (EC 3.6.1.3) and ($\text{Na}^{+} + \text{K}^{+}$)-ATPase by the method of Schoner *et al.* (1967). Lactate dehydrogenase (EC 1.1.1.27) was assayed by the method of Wróblewski & La Due (1955) with a Biochemica Test Combination kit (Boehringer).

Results

The subcellular distribution of γ -glutamyltransferase was compared in both membrane preparations with those of alkaline phosphodiesterase I, alkaline nitrophenyl phosphatase, succinate-tetrazolium oxidoreductase and lactate dehydrogenase. The alkaline phosphodiesterase I has been shown in both liver cells and lymphocytes to be a specific plasma-membrane marker (Tonster *et al.*, 1970; Abney *et al.*, 1976), and alkaline nitrophenyl phosphatase was previously demonstrated to be primarily located in the plasma membrane of thymocytes (G. Brunner & J. R. Golecki, unpublished results; Ruuskanen *et al.*, 1975; Schmidt-Ullrich *et al.*, 1974) and HeLa cells (Bosman *et al.*, 1968; Lin *et al.*, 1976). Use of either the ($\text{Mg}^{2+} + \text{Na}^{+} + \text{K}^{+}$)-ATPase or the ($\text{Na}^{+} + \text{K}^{+}$)-ATPase as plasma-membrane markers was also investigated but was not satisfactory. The ($\text{Mg}^{2+} + \text{Na}^{+} + \text{K}^{+}$)-ATPase was evenly distributed between the plasma-membrane and mitochondrial fractions, but the ($\text{Na}^{+} + \text{K}^{+}$)-ATPase activities were extremely variable, as observed by Van Blitterswijk *et al.* (1973). Attempts were also made to follow the distribution of the classic plasma-membrane marker, 5'-nucleotidase, but activities were extremely low in these plasma-membrane preparations and were due to alkaline phosphatase.

Succinate-tetrazolium oxidoreductase was determined as a marker for mitochondrial and lactate dehydrogenase for cytoplasmic material. Tables 1 and 2 summarize the results of analysis of subcellular fractions prepared by both methods. Calculated in terms of the total activity per subcellular fraction as a proportion of the summed activities of the separated fractions, the distribution of the γ -glutamyltransferase was as follows: (i) by the pump

disruption method (Table 1): 43.7% in the crude membrane fraction (C), 23.4% in the pellet of the supernatant from this fraction (D), 23.8% in the large-granule fraction (B); (ii) by the nitrogen-cavitation method (Table 2): 42% in the crude membrane fraction (I), 29.7% in fraction (H) and 19.3% in the nuclear fraction (F). It should be noted that the recovery of γ -glutamyltransferase activity was consistently greater than that measured in the cell homogenate, suggesting stimulation of the enzyme as a consequence of fractionation. In the pump method the highest specific activity was also found in the crude membrane fraction (C) and this was enriched 1.4-fold in the PM_1 band and 1.9-fold in the PM_2 band after sucrose-density-gradient purification. By the nitrogen-cavitation method, the crude-membrane fraction (I) and fraction (H) had the highest specific activities of γ -glutamyltransferase. The crude-membrane specific activity was enriched 2.5-fold in fraction S_1 after sucrose-density-gradient purification. Electron-microscopic pictures (not shown) of bands PM_1 and PM_2 and of fraction S_1 (Brunner *et al.*, 1977) showed typical plasma-membrane vesicles, free of ribosomal and mitochondrial contamination. Attempts were made with glucose 6-phosphatase and cytochrome *P*-450 to follow the subcellular distribution of the endoplasmic reticulum. However, the activity of glucose 6-phosphatase was extremely low and its distribution among plasma membranes and other fractions was not clearly defined. These observations have also been made in lymphoid cells by Van Blitterswijk *et al.* (1973) and Allan & Crumpton (1970), and they suggested that glucose 6-phosphatase is of little value as an endoplasmic-reticulum marker in these cell types. With the assay system of Burke & Mayer (1974), no detectable cytochrome *P*-450 activity could be found in calf thymocytes or their subcellular fractions. Brunner *et al.* (1977) have also observed only trace amounts of endoplasmic reticulum in calf thymocytes by using electron microscopy. The distribution of the plasma-membrane markers, alkaline phosphodiesterase I and alkaline nitrophenyl phosphatase, supports the electron-microscope evidence that PM_1 , PM_2 and S_1 fractions consisted primarily of plasma-membrane vesicles, and the absence of succinate-tetrazolium oxidoreductase and lactate dehydrogenase showed that mitochondrial and cytoplasmic contamination was negligible.

From data for both methods of preparation the increase in γ -glutamyltransferase specific activity (purification factor) with respect to the homogenate was 30.6-fold in fraction PM_1 , 42-fold in fraction PM_2 and 52.6-fold in fraction S_1 . This compares favourably with the purifications of (a) alkaline phosphodiesterase I and (b) alkaline nitrophenyl phosphatase, which were: (a) 18.7-fold for fraction PM_1 , 35.9-fold for fraction PM_2 and 20.2-fold for

Table 1. Subcellular distribution of γ -glutamyltransferase and other cell-organellar markers after cell disruption by the pump method
Results given are from a typical example of four such experiments with 5×10^{10} cells. Mean values are purposely not presented, as the variation in enzyme specific activities between different thymi was large. The percentage distribution of the enzyme activities in the subcellular fractions was, however, remarkably constant. Specific activity is nmol/min per mg of protein at 37°C. The sucrose-gradient fractions 1 and 2 and pellet were obtained by fractionation of the crude membrane (C).

	γ -Glutamyltransferase		Alkaline phosphodiesterase I		Alkaline nitrophenyl phosphatase		Succinate-tetrazolium oxidoreductase		Lactate dehydrogenase	
	Specific activity (nmol/min)	Total activity (nmol/min)	Specific activity (nmol/min)	Total activity (nmol/min)	Specific activity (nmol/min)	Total activity (nmol/min)	Specific activity (nmol/min)	Total activity (nmol/min)	Specific activity (nmol/min)	Total activity (nmol/min)
Homogenate	0.5	797	11.5	18.3	21.3	33.9	3.1	4900	565	900
Nuclear (390g _{av} × 15 min)	0.3	88	5.6	1.8	7.1	2.3	4.7	1500	81.5	26.6
Large granule (B) (4820g _{av} × 15 min)	3.0	231	57.9	4.5	89.7	6.9	35.4	2700	61.9	4.8
Crude membrane (C) (19200g _{av} × 45 min)	11.0	424	223	8.6	303	11.7	13.5	520	74.9	2.9
Supernatant (19200g _{av} fraction)	<0.05	—	4.8	3.7	9.7	7.4	0.7	540	1092	835
Supernatant pellet (D) (177000g _{av} × 90 min)	4.2	227	83.4	4.5	99.5	5.4	1.1	60	696	37.6
Sucrose-gradient fraction 1 (PM ₁)	15.3	52	327	1.1	398	1.4	0.4	1.0	0	0
Sucrose-gradient fraction 2 (PM ₂)	21.0	227	413	4.5	764	8.3	1.5	16.0	7.3	0.08
Sucrose-gradient pellet (E)	5.0	60	80	0.9	88	1.1	13.2	160	2.3	0.03
Recovery (%)		121.7		102		83.5		107.3		96.6

Table 2. Subcellular distribution of γ -glutamyltransferase and other cell-organellar markers after cell disruption by nitrogen cavitation
Conditions are as described in Table 1, except that 1×10^{11} cells were used for the experiments. The sucrose-gradient fractions were obtained by fractionation of the crude membrane (I).

	γ -Glutamyltransferase		Alkaline phosphodiesterase I		Alkaline nitrophenyl phosphatase		Succinate-tetrazolium oxidoreductase		Lactate dehydrogenase	
	Specific activity (nmol/min)	Total activity (nmol/min)	Specific activity (nmol/min)	Total activity (nmol/min)	Specific activity (nmol/min)	Total activity (nmol/min)	Specific activity (nmol/min)	Total activity (nmol/min)	Specific activity (nmol/min)	Total activity (nmol/min)
Homogenate	0.35	596	15	25.5	28.8	49	3.3	5617	618	1052
Fraction (F) (220g _{av} × 15 min)	0.48	175	8.3	3	6.9	2.5	4.2	1529	203	74
Fraction (G) (4800g _{av} × 10 min)	1.75	82	43.4	2	81	3.8	29.8	1401	139	6.6
Fraction (H) (19200g _{av} × 20 min)	6.75	270	171	6.8	386	15.4	27.6	1104	221	8.8
Crude-membrane fraction (I) (177000g _{av} × 60 min)	7.23	381	207	10.9	326	17.2	1.2	63	17.6	0.9
Crude-membrane supernatant	0	0	0.6	0.5	1.9	1.5	0.5	385	1000	770
Sucrose-gradient fraction S ₁	18.4	390	303	6.4	530	11.2	0.9	19.1	0	0
Sucrose-gradient fraction S ₂	0	0	30.9	0.95	45	1.4	1.4	43	23.7	0.7
Sucrose-gradient fraction S ₃	0	0	39.9	0.2	58	0.3	1.4	8	15.1	0.08
Recovery (%)		152		91		82.4		80		81.8

fraction S₁; (b) 28.4-fold for fraction PM₁, 35.9-fold for fraction PM₂ and 18.4-fold for fraction S₁. This distribution of the γ -glutamyltransferase strongly suggests that it is primarily a plasma-membrane-bound enzyme in calf thymocytes.

The exact localization of the γ -glutamyltransferase activity appearing in other subcellular fractions is at present not entirely clear. That portion co-pelleting with the nuclear (F) and large-granule (mainly mitochondrial) fractions (B) and (H) may also be plasma-membrane-bound, since electron microscopy showed the presence of large membrane vesicles as well as mitochondria in fractions (B) and (H), whereas much membranous material is trapped in the nuclear pellet, after cell disruption is achieved by nitrogen cavitation. γ -Glutamyltransferase activity found in the sucrose-gradient pellet (E) and in pellet (D) may also be plasma-membrane-bound because electron microscopy revealed a mixture of some membrane vesicles, some mitochondria and much ribosomal material in pellet (E), whereas pellet (D) contained small plasma-membrane vesicles in addition to much ribosomal material. The relatively high total contents of both alkaline phosphodiesterase I and alkaline nitrophenyl phosphatase activity in pellet (D) support this idea.

Discussion

The above demonstration, by using two different methods of cell disruption and plasma-membrane isolation, that γ -glutamyltransferase is primarily located in the plasma membrane of calf thymocytes is also in agreement with its location in rabbit erythrocytes (Palekar *et al.*, 1974). In rat kidney, γ -glutamyltransferase is also mainly located in brush-border membranes, but at least 25% of the total activity is recovered in the mitochondrial fraction (Hughes *et al.*, 1978). The subcellular distribution of γ -glutamyltransferase in calf thymocytes as shown here is consistent with the suggestion of Novogrodsky *et al.* (1976) that this enzyme is located on the cell surface of lymphoid cells. Although it cannot be excluded that 25–30% of the measured activity could be localized in mitochondria, we felt that it is more likely to be bound to membrane vesicles co-pelleting with this fraction.

There is at present much speculation as to the physiological role of γ -glutamyltransferase. Whereas Meister & Tate (1976) believe that, in conjunction with the γ -glutamyl cycle, this enzyme is primarily concerned with amino acid transport in kidney, Elce & Broxmeyer (1976) argue that this role is most unlikely according to enzyme kinetic data and proposed that γ -glutamyltransferase in the kidney is more probably concerned with hydrolysis of oxidized glutathione as a means of avoiding the loss of amino acid residues into the urine. Pellefigue *et al.*

(1976) have also shown in cultured human fibroblasts, from a patient deficient in γ -glutamyltransferase activity, that this enzyme is not involved in amino acid transport.

Novogrodsky *et al.* (1976) observed mitogen stimulation of γ -glutamyltransferase in various cultured lymphoid cells, but the earliest times reported to produce stimulation were after 21 h of culture in the presence of the mitogen.

It is therefore possible that γ -glutamyltransferase could be associated with amino acid uptake in mitogen-stimulated lymphocytes at a later stage, providing amino acids for protein synthesis during the cell-proliferative phase, but that has yet to be investigated.

The demonstration here that γ -glutamyltransferase, which is readily determined, is mainly located in the plasma-membrane fractions of calf thymocytes provides a new plasma-membrane marker enzyme for lymphoid cells.

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