RAPID PURIFICATION OF RAT KIDNEY BRUSH BORDERS ENRICHED IN γ-GLUTAMYL TRANSPEPTIDASE

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1. Introduction

The proximal tubule cells of the kidney are characterized by the presence of numerous, closely packed microvilli on their luminal surface, the so-called brush border, and by interdigitated processes of the basal-lateral plasma membrane on the contraluminal side. These morphologic differences are accompanied by uneven distribution of various transport processes and enzyme activities in these two membrane structures. Thus, the basal-lateral membranes are enriched in Na^+-K^+-ATPase and Ca^{2+}-ATPase. The brush-border membranes are characterized by high activities of various esterases, disaccharidases, and peptidases, and exhibit Na^+-dependent transport of various metabolites [1]. Basal-lateral and brush-border membranes have been separated with varying degree of success employing methods based on differences in size and density, e.g., sedimentation and centrifugation methods, and differences in surface charges [2–4]. Relatively pure basal-lateral membranes and microvilli have been obtained [4] by a free-flow electrophoresis technique. Many of these methods are time-consuming or require elaborate equipment. As part of our continuing studies on γ-glutamyl transpeptidase the brush-border enzyme that catalyzes the first step in glutathione utilization and is believed to mediate amino acid and/or peptide transport [5–7], we have devised a simple method, described here, for rapid separation of rat renal basal-lateral membranes from microvilli. The method involves treatment of the crude brush-border fraction (the pre-free flow membrane fraction in [4]) with relatively low concentrations of $MnCl_2$. This selectively affects the sedimentation behaviour of the basal-lateral membranes, which can then be readily separated from microvilli by low-speed centrifugation.

2. Materials and methods

L-γ-Glutamyl-*p*-nitroanilide, *p*-nitrophenylphosphate, *S*-benzyl-L-cysteinyl-*p*-nitroanilide, glycylglycine, ATP, and papain (18 units/mg) were purchased from Sigma. L-γ-Glutamyl-(4-methoxy)-2-naphthylamide and 4-aminophthalhydrazide were obtained from Polysciences. All spectrophotometric assays were performed at 37°C in a Cary Model 15 spectrophotometer. Centrifugations were carried out in a Sorvall RC-5 centrifuge.

The activities of marker enzymes were determined as described below (with associated references): γ-glutamyl transpeptidase was assayed in presence of 1 mM γ-glutamyl-*p*-nitroanilide and 20 mM Gly–Gly [8]. Dipeptidase activity was determined as in [9] with *S*-benzyl-L-cysteinyl-*p*-nitroanilide. Alkaline phosphatase activity was measured in a solution containing 2 mM *p*-nitrophenylphosphate, 5 mM $MgCl_2$, 2.5 mM NaF and 75 mM glycine buffer (pH 9.5) [10]. Succinate dehydrogenase was assayed as in [11], using 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyletrazolium chloride (INT) as the electron acceptor. Na^+-K^+-ATPase was assayed by a modification of the technique in [12]. The membranes were incubated with 0.1% sodium deoxycholate for 30 min at 25°C and then assayed in a solution containing 6 mM Tris–ATP, 12 mM KCl, 120 mM NaCl, 12.5 mM $MgSO_4$, 5 mM NaN_3, 0.4 mM EDTA and 25 mM Tris–HCl buffer (pH 7.3). Control consisted of a solution containing no KCl and to which 2 mM

Table 1
Enzyme activities in rat kidney homogenate and membrane fractions

Fraction	Protein		γ-Glutamyl transpeptidase			Alkaline phosphate		Dipeptidase		Na^+-K^+-ATPase		Succinate dehydrogenase	
	Total (mg)	Yield (%)	Total (units)	Yield (%)	Specific activity	Yield (%)	Specific activity	Yield (%)	Specific activity	Yield (%)	Specific activity	Yield (%)	Specific activity
Homogenate	1280	100	2005	100	1.6 (1)	100	0.7 (1)	100	0.3 (1)	100	2.3 (1)	100	1.1
Fraction P_5	42	3.3	496	24.7	11.9 (7.6)	23.6	5.4 (7.7)	16.5	1.7 (5.8)	11	7.8 (3.4)	0.4	0.2
Fraction P_5A	24.6	1.9	49	2.4	2.0 (1.3)	2.9	1.1 (1.6)	4.8	0.7 (2.4)	8.2	9.8 (4.3)	0.2	–
Fraction P_5B	17	1.3	354	17.6	20.8 (13.2)	16.8	9.4 (13.4)	9.3	3.2 (10.5)	1.2	4.1 (1.8)	0.1	–

P_5, pre-free flow electrophoresis fraction from [4]; P_5A, membrane fraction sedimentable at 750 × *g* after $MnCl_2$ treatment (basal-lateral membranes); P_5B, membrane fraction whose sedimentation behaviour was unaffected by $MnCl_2$ treatment (brush-border membranes; fig.1C)

Specific activities of the various enzymes are expressed as follows: γ-glutamyl transpeptidase and alkaline phosphatase, μmol *p*-nitroaniline and *p*-nitrophenol released/min/g protein, respectively; dipeptidase, μmol *p*-nitroaniline produced/h/mg protein; Na^+-K^+-ATPase, μmol inorganic phosphate released/h/mg protein; succinate dehydrogenase, μmol INT formazan produced/h/mg protein. Numbers in parenthesis represent relative specific activity (homogenate = 1)

oubain was added. Reactions were terminated with trichloroacetic acid and phosphate determined in protein-free supernatant [13]. Protein was determined as in [14].

Ultrastructural localization of γ-glutamyl transpeptidase in membrane preparation was carried out as in [15]. The membranes were incubated with γ-glutamyl-(4-methoxy)-2-naphthylamide and Gly–Gly in presence of freshly diazotized 4-aminophthalhydrazide. An insoluble, lipophobic, and osmiophilic azo dye is produced at the site of enzyme activity. The osmicated and dehydrated samples were embedded in Araldite and ultrathin sections cut on a LKB ultramicrotome, stained with lead citrate, and observed in a JEM-100B electron microscope. In controls, L-serine and borate (5 mM each), a combination known to inhibit transpeptidase [16,17] were included in the incubation medium.

3. Results

The kidney tissue from male Sprague-Dawley rats (300 g) was homogenized and fractionated according to the scheme in [4] up to the pre-free flow electrophoresis stage (fraction P_5). Homogenization buffer consisted of 10 mM triethanolamine–HCl (pH 7.5) containing 0.25 M sucrose (TS buffer). Fraction P_5, obtained after several centrifugation steps, represents a crude membrane fraction rich in both the luminal (brush-border) and contraluminal (basal-lateral) membranes. These membranes sediment at 16 000 × *g* as a white, fluffy layer above the mitochondrial pellet. Further fractionation of P_5 was carried out as follows: Aliquots of fraction P_5 were adjusted to ~1 mg protein/ml with TS buffer and $MnCl_2$ was added to give final conc. 2 mM. After incubation for 2 h at 0°C, the suspension was centrifuged at

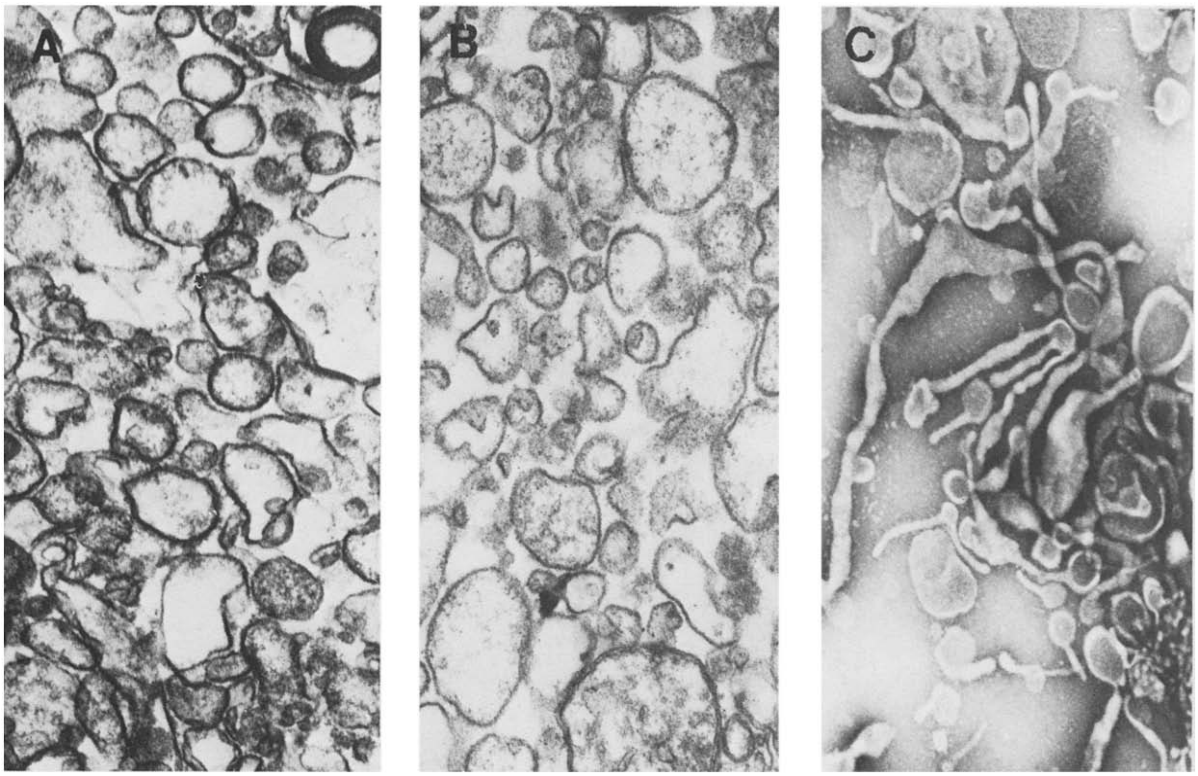

Fig.1. Ultrastructural demonstration of γ-glutamyl transpeptidase activity in fraction P_5B (A). Note the thicker and darker appearance of membranes in this micrograph of thin sections indicative of transpeptidase activity compared to the appearance of membranes in the control (B) in which transpeptidase was inhibited; × 50 000. (C) Fraction P_5B negatively-stained with 2% phosphotungstate (pH 7.5) on a Formvar-coated grid. Note the numerous microvilli; × 50 000.

750 × *g* for 20 min. The pellet was resuspended in TS buffer (using a loose-fitting Dounce homogenizer) and centrifuged as above. The pellet was suspended in a small volume of TS buffer (fraction P_5A). The supernatants from the above treatments were combined, centrifuged at 16 000 × *g* for 40 min, and the pellet obtained was suspended in TS buffer (fraction P_5B).

Results of a fractionation are given in table 1. Fraction P_5A exhibited a relatively high specific activity of Na^+-K^+-ATPase, a marker enzyme for basal-lateral membranes. On the other hand fraction P_5B, whose sedimentation behavior remains unaffected by $MnCl_2$ treatment, was characterized by relatively high specific activity of brush-border enzymes, alkaline phosphatase and γ-glutamyl transpeptidase. In separate experiments, fraction P_5B generally exhibited a 12–15-fold increase in specific activity of these two enzymes over the homogenate. It is of interest that dipeptidase (assayed as described and which appears to be a broad-specificity enzyme [19]) is enriched about 11-fold in P_5B. Contamination of both fractions P_5A and P_5B by other organelles (e.g., mitochondria) was negligible.

Effect of varying $MnCl_2$ concentration (from 0.5–5 mM) and temperature on the fractionation was studied. Efficiency of other metal salts (e.g., $MgCl_2$ and $CaCl_2$) was also investigated. The best fractionation was achieved with $MnCl_2$, described above.

Negative staining showed that fraction P_5B consisted of nearly pure, morphologically intact microvilli (fig.1C). Figure 1A shows that almost all vesicles seen in ultra-thin sections of this fraction exhibit γ-glutamyl transpeptidase activity localized to the membranes (as indicated by thicker and darker appearance of these membranes compared to the appearance of the membranes in the corresponding control (fig.1B)). Similar treatment of P_5A showed that ~10% of the membrane vesicles in this preparation stained positively for transpeptidase activity; however, negative staining of this fraction showed almost complete absence of intact microvilli (data not shown).

4. Discussion

The fractionation procedure described here is particulary useful in rapid isolation of morphological intact microvilli. These membranes, which retain their original sedimentation behavior, are enriched upto 15-fold in enzymes such as γ-glutamyl transpeptidase and a dipeptidase capable of hydrolyzing cysteinyl glycine [9]. Studies are now in progress on the topology and function of these glutathione-utilizing enzymes in the microvilli. Preliminary experiments indicate that almost all intact microvilli can be labeled with a ferritin–concanavalin A conjugate indicating that they have right-side out configuration. Papain treatment of intact microvilli solubilized ~60% of transpeptidase and no alkaline phosphatase (unpublished data). Partial solubilization (~60%) of transpeptidase from intact kidney cells [18] and brush-border membranes by papain [19,20] has been noted by other workers as well. Alkaline phosphatase, an integral membrane protein, is not solubilized [20]. Thus, the brush borders, isolated as described here, exhibit properties similar to preparations described by others.

The basis for changes in the sedimentation behavior of the basal-lateral plasma membranes following treatment with $MnCl_2$ is unknown at present. Further studies are required to clarify the processes involved. The differences in the behavior of the two membrane fractions following Mn treatment emphasizes the differences in their structure and composition.

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