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Molecular size of the renal sodium/phosphate symporter in native and reconstituted systems

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The size of the renal sodium/phosphate symporter was estimated with the radiation inactivation technique in isolated bovine brush border membrane vesicles and after reconstitution in proteoliposomes. The functional unit of the native phosphate carrier had a radiation inactivation size of 172 ± 17 kDa. Identical values were obtained for the reconstituted carrier whether it was irradiated before or after the formation of the proteoliposomes (161 ± 9 and 159 ± 11 kDa, respectively). The sodium-independent uptake of phosphate was not affected significantly by radiation doses up to 10 Mrad. This activity is therefore not due to the reconstitution of a large phosphate-binding protein such as alkaline phosphatase. Furthermore, bromotetramisole, a specific inhibitor of phosphate binding to this enzyme, had no significant effect on the uptake of phosphate by the proteoliposomes.

Introduction

Reabsorption of phosphate from the glomerular filtrate involves a sodium/phosphate symporter located in the luminal membrane of the epithelial cells lining the proximal tubule [1–6]. Despite extensive studies on the transport of phosphate across the renal brush border membrane, the symporter protein that carries out this activity remains to be identified. As a first step towards the isolation of this transporter, we have recently developed a reconstitution protocol that allows one to monitor its activity after solubilization of the brush border membrane [7]. The reconstituted phosphate carrier retains the main characteristics of the native carrier, including a strong sodium-dependent transport activity. However, our proteoliposomes also take up substantial amounts of phosphate in the absence of sodium [7]. This sodium-independent phosphate uptake activity could be due to the reconstitution of a damaged or incomplete carrier or to the presence of phosphate-binding proteins such as alkaline phosphatase [8], phosphorin [9] or another recently described proteolipid [10] extracted from the brush border membrane.

The present study was undertaken to distinguish between these possibilities and to ascertain the nature of the molecules responsible for both the sodium-dependent and the sodium-independent reconstituted phosphate uptake activities. Radiation inactivation analysis [11–13] yielded a similar target size for sodium-dependent phosphate transport in brush border membrane vesicles and in reconstituted proteoliposomes. In contrast, the sodium-independent phosphate uptake was unaffected by irradiation, at doses ranging from 0 to 10 Mrad, and could therefore not be due to the reconstitution of a large protein. In particular, alkaline phosphatase, although it was partially reconstituted, does not appear to play a significant role since bromotetramisole, a potent inhibitor of its phosphate-binding activity, was unable to influence phosphate uptake by the proteoliposomes.

Materials and methods

Preparation of brush border membrane vesicles

precipitation method [14] as described previously [15]. The mean enrichment for alkaline phosphatase was 6.9 ± 1.3. Prior to irradiation, the membranes were washed and resuspended in a cryoprotective medium containing 150 mM KCl, 14% (w/v) glycerol, 1.4% (w/v) sorbitol, 5 mM Hepes-Tris (pH 7.5), and stored at −80°C until use.

Reconstitution protocol

The procedure used for the reconstitution of the phosphate carrier has already been described in detail [7]. Briefly, brush border membrane proteins were extracted in a buffer containing brush border membrane lipids (10.2 μmol phospholipids/ml), 30% (w/v) glycerol, 1.5% (w/v) CHAPS, 150 mM Na_2HPO_4-NaH_2PO_4, 5 mM Hepes-Tris (pH 7.5), and 1 mM dithiothreitol. The mixture was centrifuged at 100 000 × *g* for 30 min and 0.5 ml of the supernatant was mixed with brush border membrane lipids (7.5 μmol phospholipids) resuspended in 0.5 ml of 150 mM Na_2HPO_4-NaH_2PO_4 (pH 7.5). Proteoliposomes were obtained after removal of the detergent by chromatography on a Sephadex G-50 column eluted with column buffer composed of 150 mM KNO_3 and 5 mM Hepes-Tris (pH 7.5). The turbid fractions eluting in the void volume were pooled and frozen in liquid nitrogen. After thawing at room temperature, the proteoliposomes were concentrated by centrifugation at 45 000 × *g* for 20 min and resuspended in column buffer. Proteoliposomes to be irradiated were washed and resuspended in the cryoprotective medium. Finally, after the irradiation, the proteoliposomes were washed and resuspended in column buffer.

Irradiation procedure

Irradiation was carried out in a Gammacell Model 220 instrument at a dose rate of approximately 1.5 Mrad/h [16]. During this procedure, the samples were maintained in dry ice. The radiation inactivation size (RIS) was calculated using the empirical equation [12]:

$$\log \mathrm{RIS} = 5.89 - \log D_{37,T} - 0.0028T$$

where $D_{37,T}$ is the radiation dose (in Mrad) necessary to inactivate the carrier protein to 37% of its initial activity, and T is the temperature (in °C). The $D_{37,T}$ values were obtained from semi-logarithmic plots of uptake versus irradiation dose using a least-squares fit. Because slightly different radiation doses were used in different experiments, the data shown in the figures are derived from representative experiments. The radiation inactivation size estimates, however, are based on at least three independent experiments.

of 5 μl of brush border membrane vesicles (35 mg protein/ml) or proteoliposomes (3–4 mg protein/ml) to 25 μl of the appropriate incubation medium as described in the figure legends. After different times of incubation, the reaction was stopped by dilution with 1 ml of an ice-cold stop solution. The suspension was filtered immediately under vacuum through a nitrocellulose filter (0.45 μm pore diameter). The filter was rinced with an additional 7 ml of stop solution and the radioactivity was determined by liquid scintillation counting. The stop solution used with brush border membrane vesicles had the same composition as the cryoprotective medium described above. Uptake by proteoliposomes was stopped with 150 mM KCl, 5 mM Hepes-Tris (pH 7.5).

Other methods

Brush border membrane lipids were extracted with the method described by Kates [18]. Lipid phosphorus was estimated with the ashing technique of Ames and Dubin [19]. Alkaline phosphatase was assayed by measuring the hydrolysis of *p*-nitrophenyl phosphate [20] and protein content was estimated with the bicinchoninic acid assay [21].

Chemicals

[^{32}P]Orthophosphate (carrier-free) was purchased from ICN Biomedicals, the bicinchoninic assay reagents, from Pierce, and (−)-*p*-bromotetramisole oxalate, from Aldrich.

Results

The molecular size of the bovine sodium/phosphate symporter was first measured in irradiated brush border membrane vesicles. In the presence of a sodium gradient, there was a progressive loss of transport activity with increasing radiation dose (Fig. 1A). In contrast, the sodium-independent phosphate uptake was not affected at the radiation doses used. The radiation inactivation size, estimated from a semi-logarithmic plot of the sodium-dependent transport activity (Fig. 1B), was 172 ± 17 kDa ($n = 4$). Phosphate transport was also measured in reconstituted proteoliposomes subjected to irradiation (Fig. 2) and in proteoliposomes prepared from irradiated brush border membrane vesicles (Fig. 3). In both cases, increasing the radiation dose from 0 to 10 Mrad resulted in a gradual decrease in the sodium-dependent transport activity, but had no significant effect on the sodium-independent uptake of phosphate. The radiation inactivation size of the sodium/phosphate symporter, as measured in irradiated proteoliposomes (Fig. 2), was 159 ± 11 kDa ($n = 3$). A similar value (161 ± 3 kDa, $n = 4$) was

Any direct role of alkaline phosphatase in mediating sodium-dependent transport of phosphate across the renal brush border membrane has been ruled out conclusively [22–26]. This enzyme does, however, bind inorganic phosphate with high affinity [8]. It was therefore important to determine to what extent it is reconstituted into our proteoliposomes and to evaluate the contribution of this binding activity to the reconstituted sodium-independent uptake of phosphate. Only 56% of the initial alkaline phosphatase activity was found in the solubilized protein fraction and 20% was recovered in the proteoliposomes. Its specific activity in proteoliposomes (459 ± 11 nmol/min per mg) was nevertheless comparable to that originally present in brush border membranes (578 ± 30 nmol/min per mg). However, bromotetramisole, which inhibits both the catalytic and phosphate-binding activities of alkaline phosphatase [8], had no significant effect on the uptake of phosphate by the proteoliposomes (Fig. 4).

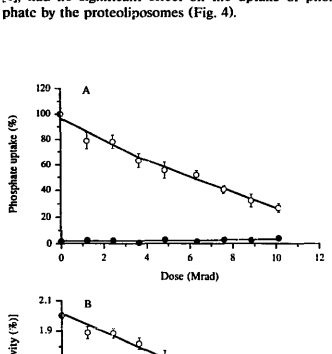

Fig. 1. Determination of the molecular size of the sodium/phosphate cotransporter in irradiated vesicles. (A) Irradiated vesicles were incubated for 5 s at 25°C in a solution containing 200 μM [^{32}P]KH_2PO_4-K_2HPO_4, 14% glycerol, 14% sorbitol, 5 mM Hepes-Tris (pH 7.5), and 150 mM NaCl (○) or 150 mM KCl (●). (B) Sodium-dependent phosphate transport was calculated as the difference between the uptake in the presence of NaCl and KCl and expressed as the log of the percentage of the activity remaining vs. the radiation dose. Each value is the mean ± S.D. of a representative experiment where each assay was performed in triplicate.

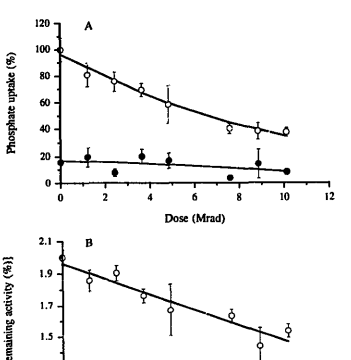

Fig. 2. Determination of the molecular size of the sodium/phosphate cotransporter in irradiated proteoliposomes. (A) Irradiated proteoliposomes were incubated for 15 min at 25°C in a solution containing 200 μM [^{32}P]KH_2PO_4-K_2HPO_4, 5 mM Hepes-Tris (pH 7.5), and 150 mM $NaNO_3$ (○) or 150 mM KNO_3 (●). (B) Sodium-dependent phosphate transport was calculated as the difference between the uptake in the presence of $NaNO_3$ and KNO_3 and expressed as the log of the percentage of the activity remaining vs. the radiation dose. Each value is the mean ± S.D. of a representative experiment where each assay was performed in triplicate.

Discussion

Similar radiation inactivation sizes were obtained for the renal sodium/phosphate symporter when estimated in irradiated brush border membrane vesicles (172 ± 17 kDa), irradiated proteoliposomes (159 ± 11 kDa), and proteoliposomes prepared from irradiated brush border membrane vesicles (161 ± 9 kDa). The sodium-dependent transport activity measured in the proteoliposomes thus results from the reconstitution of an apparently intact carrier protein. These results suggest that the carrier molecule, which is thought to consist of an oligomeric protein [27], retains its native structure as well as its functional properties upon reconstitution [7]. The values reported here for the bovine phosphate carrier are smaller but comparable to those previously published for the renal phosphate carriers of the rat (205 ± 14 kDa) [28] and the mouse (242 ± 16 kDa) [29]. The significance of this variation among species remains to be established, although it is per-

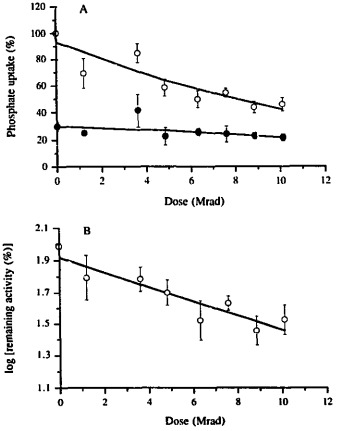

Fig. 3. Determination of the molecular size of the sodium/phosphate cotransporter in proteoliposomes prepared from irradiated vesicles. (A) The proteoliposomes were incubated for 15 min at 25°C in a solution containing 200 μM [^{32}P]KH_2PO_4-K_2HPO_4, 5 mM Hepes-Tris (pH 7.5), and 150 mM $NaNO_3$ (○) or 150 mM KNO_3 (●). (B) Sodium-dependent phosphate transport was calculated as the difference between the uptake in the presence of $NaNO_3$ and KNO_3 and expressed as the log of the percentage of the activity remaining vs. the radiation dose. Each value is the mean ± S.D. of a representative experiment where each assay was performed in triplicate.

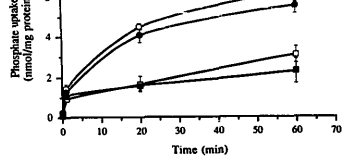

Fig. 4. Effect of bromotetramisole on the uptake of phosphate by reconstituted proteoliposomes. The proteoliposomes were incubated at 25°C in a solution containing 200 μM [^{32}P]KH_2PO_4-K_2HPO_4, 5 mM Hepes-Tris (pH 7.5), and 150 mM $NaNO_3$ (○, ●) or KNO_3 (□, ■) in the presence (●, ■) or absence (○, □) of 1 mM (−)-*p*-bromotetramisole.

haps not surprising in view of their different diets and digestive physiologies, since the target size for phosphate transport in rat kidney was previously reported to be affected by the phosphate content of the diet [27]. In contrast with the sodium-dependent transport activity, the uptake of phosphate measured in the absence of sodium was not affected significantly by radiation doses ranging from 0 to 10 Mrad. The relatively strong sodium-independent phosphate uptake by proteoliposomes is therefore not due to the reconstitution of a large protein such as the phosphate carrier itself or alkaline phosphatase which has a radiation inactivation size of 69 kDa in calf kidney brush border membranes [30]. Binding of phosphate to alkaline phosphatase is also excluded in view of the lack of effect of bromotetramisole on the uptake of phosphate by the proteoliposomes.

The structural identity of the renal sodium/phosphate symporter remains elusive. In particular, the role of phosphate-binding proteolipids extracted from the brush border membrane [9,10] is still unclear. Since these compounds were extracted with mixtures of chloroform and methanol, they may be present in the lipid extract used for the preparation of our proteoliposomes. Phosphorin [9], a 3 kDa polypeptide, is too small to become inactivated at the small doses used in the present study. Although it could contribute to the sodium-independent binding of phosphate, purified phosphorin reconstituted into proteoliposomes did not mediate sodium-dependent transport of this ion [31]. Another brush border membrane proteolipid, which appears to exist as a dimer of about 155 kDa, was shown to bind sodium and phosphate [10]. This protein is probably not involved in the sodium-independent uptake of phosphate by our proteoliposomes since it would have been inactivated readily at the doses used. Although its size is similar to that of the sodium/phosphate symporter, liposomes prepared with total lipids extracted from the brush border membrane, but without added protein, were unable to take up phosphate in a sodium-dependent manner [7]. Further work will clearly be required to resolve the important question of the molecular identity of the renal sodium/phosphate symporter. Recent progress in the functional reconstitution of this protein [7] and in the cloning of its gene [32] should help to make this an attainable goal.

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