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Biochimica et Biophysica Acta 1419 (1999) 23–32

BIOCHIMICA ET BIOPHYSICA ACTA
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Purification and characterization of a major zinc binding protein from renal brush border membrane of rat

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Received 8 December 1998; received in revised form 16 March 1999; accepted 25 March 1999

Abstract

In spite of the fact that zinc is an essential trace element, mechanisms that contribute to zinc homeostasis in mammals are poorly understood. An attempt has been made to identify and purify zinc binding components from renal brush border membrane (BBM), which could be involved in the binding of zinc and the subsequent translocation across the BBM. A 40 kDa major zinc binding protein has been identified and purified from renal BBM, which showed a dissociation constant (K_d) of 211 μM and maximal binding (B_{max}) of 207 nmol/mg protein. 8 g zinc atoms could interact with 1 mol of protein. Specificity of the protein for zinc was checked by metal displacement and UV-absorption assay. It was found that only Cd^{2+} could displace the zinc bound to the protein. Other metals tested (Cu^{2+}, Mg^{2+}, Ca^{2+}) did not show any interaction with the protein. These data indicated that purified protein is highly specific and has a high affinity for zinc. The carbohydrate content was found to be 7.85 mg% in the purified protein. Immunofluorescence localization of this protein in kidney sections revealed that this major zinc binding protein is exclusively localized in the proximal convoluted tubules. These results suggested that the 40 kDa major zinc binding transmembrane glycoprotein is highly specific for zinc and has a high affinity for zinc. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Transmembrane zinc binding protein; Kinetic constant; Rat kidney cortex; Metal-protein interaction

1. Introduction

Although zinc is an essential component of a wide variety of metalloenzymes, transcription factors and other proteins, mechanisms that contribute to zinc homeostasis in mammals are poorly understood [1]. Zinc is a small hydrophilic and charged species which cannot cross biological membranes by passive diffusion. Therefore specialized mechanisms are required for both its uptake and release [2]. The transport of zinc has been studied in several different isolated cell types including hepatocytes [3–5], fibroblasts [6], intestinal cells [7], endothelial cells [8] and membrane vesicles [9–12]. Although differences exist in zinc transport in these systems, all exhibit some common characteristics. Zinc transport is a temperature- and pH-sensitive process and there appear to be both saturable and non-saturable components [13]. However, there may also be an energy requirement for some aspects of zinc transport. Although the kinetic characteristics of zinc transport have been studied in a wide variety of model systems, proteins directly associated with zinc transport had not been described until recently. Recently four genes involved in mammalian zinc transport have been cloned. These all predict proteins with multiple membrane spanning

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PII: S0005-2736(99)00054-1

domains and most have a histidine rich intracellular loop. Zinc transporter-1 (ZnT-I) was first cloned and is associated with efflux in the baby hamster kidney (BHK) cell line [14]. It is found in all tissues examined, and at least in some of the tissues ZnT-I expression is regulated by dietary zinc intake. In enterocytes and renal tubular cells, ZnT-I is localized to the basolateral membrane, suggesting an orientation that is consistent with the zinc acquisition and/or retention function. ZnT-2 is also an exporter and may be involved in zinc efflux or uptake into vesicles (lysosomal/endosomal) in the intestine, kidney and testes [15]. ZnT-3 is involved in zinc uptake into synaptic vesicles in neurons and possibly in testes [16]. ZnT-4 is also an exporter and is highly expressed in the mammary glands and brain [17]. Strikingly, none of the transporter has been shown to be involved in the influx of zinc into the cells.

The first barrier in the zinc uptake is the brush border membrane (BBM) in the renal tubular cells (Fig. 1). Some carrier proteins have to be involved in the transport of highly charged species across this membrane. Studies from our laboratory on zinc uptake into the BBM vesicles prepared from purified renal BBM have revealed the presence of extravesicular as well as intravesicular zinc binding sites [12]. Further studies were conducted to purify and characterize the zinc binding components from renal BBM which could be putative transporters of zinc from the lumen into the cells across BBM.

2. Materials and methods

2.1. Animals

Young male rats of the wistar strain, weighing 150–200 g, were obtained from the animal breeding colony of the institute. The animals were acclimatized to laboratory conditions before commencement of the experiment. The animals were kept under hygienic conditions and fed a standard rat pellet diet (Ashirbad, Chandigarh, India) and were given water ad libitum.

2.2. Chemicals

$^{65}ZnCl_2$ (specific activity 532.3 mCi/g) was purchased from Bhabha Atomic Research Centre (Trombay, Mumbai, India). *N*′-2 Hydroxyethylpiperazine, *N*-2-ethane-sulfonic acid (HEPES), *n*-octylglucoside, NP-40, lubrol px, phenyl-Sepharose-4B, ethylene glycol bis-β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), silver nitrate, TEMED were purchased from Sigma (St. Louis,MO, USA), Sephadex G100 and DEAE-Sephadex were obtained from Bio-Rad (Mississauga, Ont., Canada). All other chemicals were analytical reagent grade compounds, obtained from standard commercial sources.

2.2.1. Preparation of renal brush border membrane

The animals were killed under light ether anesthesia. Rat renal cortical BBM were isolated by differential centrifugation method of Beck and Sacktor [18] and Prasad et al. [12]. The cortices from kidneys were dissected and 10% homogenate was made in ice cold 50 mM mannitol buffered with 15 mM HEPES buffer (pH 7.0). All preparative steps were carried out at 4°C. $MnCl_2$ was added to a final concentration of 4 mM and the mixture was stirred in an ice bath for 10 min. The suspension was centrifuged at $4000 \times g$ for 15 min. The supernatant so obtained was centrifuged at $43\,000 \times g$ for 20 min. The pellet was suspended in 300 mM mannitol, 25 mM HEPES buffer (pH 6.9) and homogenized three times manually. The suspension was centrifuged again at $43\,000 \times g$ for 20 min. The resulting pellet was resuspended in 300 mM mannitol, 25 mM HEPES buffer (pH 6.9). This step was repeated twice and finally the pellet was resuspended in 300 mM mannitol, 25 mM HEPES buffer (pH 6.9). The purity of the BBM was checked by assaying marker enzymes of BBM in homogenate and finally isolated BBM preparation.

Alkaline phosphatase (EC 3.1.3.1) was assayed by monitoring the production of *p*-nitrophenol from *p*-nitrophenyl phosphate [19] and maltase (EC 3.2, 1.20) activity was determined by measuring D-glucose liberated from maltose using the glucose oxidase-peroxidase system of Dahlqvist [20]. Contamination from the basolateral membrane was checked by assaying Na^+/K^+ ATPase (EC 3.6.1.3), which was measured by the method of Quigley and Gotterer [21]. Protein content was determined by the method of Bradford [22]. Alkaline phosphatase and maltase were found to be enriched about 10–13-fold in the BBM preparation. There was negligible contamina-

tion of the basolateral membrane as checked by assaying its marker enzyme Na^+/K^+ ATPase. Only high purity BBM was used for further studies.

2.3. Purification of zinc binding protein

2.3.1. Solubilization of BBM proteins

An appropriate amount of BBM protein (5.0 mg/ml) was incubated at 4°C for 1 h in a reaction mixture containing 1 mM Zn^{2+} in 25 mM HEPES, 100 mM mannitol buffer (pH 7.0) and 10 μCi of $^{65}Zn^{2+}$. Then it was incubated with 1% *n*-octylglucoside (protein to detergent ratio 1:4 w/w) at 4°C and the reaction mixture was stirred for 1 h. Solubilized proteins were separated from insoluble material by centrifugation at $40\,000 \times g$ for 1 h. $80.54 \pm 5.4\%$ of the protein was recovered on solubilization. The solubilized protein sample was dialysed for 10–12 h in a dialysis bag (10 kDa cut off). The solubilized protein was subjected to the following chromatographic separation procedure.

2.3.2. Ion exchange chromatography

The dialyzed protein sample was concentrated to 3.0 ml using Amicon ultrafiltration assembly with a 10 kDa cut off membrane filter. It was charged on to a DEAE-Sephadex A-25 column (12 cm × 1.2 cm), pre-equilibrated with 20 mM Tris buffer (pH 7.0) containing 1% lubrol px. Unbound proteins were removed by washing the column with the same buffer (3× bed volume). Bound proteins were eluted with a linear ionic gradient formed by mixing 100.0 ml each of 20 mM Tris-HCl buffer containing 1.0% lubrol px and 1.0 M NaCl in the same buffer. 3.0 ml fractions were collected at a flow rate of 12 ml/h and monitored for absorbance at 280 nm and radioactivity of ^{65}Zn was measured as counts per min (CPM) in a gamma-scintillation counter (1282 Compugamma universal γ counter). Fractions showing high zinc binding specific activity were pooled, desalted and concentrated by ultrafiltration.

2.3.3. Molecular exclusion chromatography

The concentrated pool of partially purified zinc binding protein from DEAE-Sephadex column was chromatographed on a Sephadex G100 column (85 cm × 1.4 cm), pre-equilibrated with 25 mM Tris buffer (pH 7.0) containing 1% lubrol px. Proteins were eluted with the same buffer at a flow rate of 8 ml/h. Fractions of 2.0 ml were collected and monitored for absorbance at 280 nm and CPM. Fractions showing high zinc binding specific activity were pooled and incubated with Biobeads SM-2 at a concentration of 10 mg/ml to remove the detergent from the protein sample.

After filtration through a syringe filter the protein sample was concentrated to 1.0 ml in an Amicon ultrafiltration assembly using a 10 kDa cut off membrane.

2.3.4. Hydrophobic interaction chromatography

A concentrated protein sample from the Sephadex G100 column was loaded onto the phenyl-Sepharose column (8 cm × 0.8 cm), pre-equilibrated with 25 mM Tris buffer (pH 7.0). Unbound protein was removed by washing the column (3× bed volume) with the same buffer. Bound proteins were eluted with a linear gradient formed by mixing 25 ml of 2% *n*-octylglucoside in 25 mM Tris buffer and 25 mM Tris buffer (pH 7.0). 800 μl fractions were collected at a flow rate of 4.0 ml/h and monitored for absorbance at 280 nm and CPM. Fractions showing high zinc binding specific activity were pooled, detergent was removed by adding biobeads SM-2 (10 mg/ml) and concentrated in an Amicon ultrafiltration assembly using a 10 kDa cut off membrane filter. Purity and homogeneity of this purified protein was checked by SDS-PAGE and fast protein liquid chromatography (FPLC).

2.3.5. Fast protein liquid chromatography

A Mono Q HR 5/5 (Pharmacia) column (5 × 50 mm) was used to check the purity of purified protein obtained after phenyl sepharose column chromatography. The Mono Q column was equilibrated with starting buffer (10 mM Tris-HCl, pH 8.0). 500 μl of sample (10 μg protein) was applied onto the Mono Q column. The column was washed with the starting buffer. The bound proteins were eluted with a linear gradient of chloride ions (0–1 M NaCl) in 10 mM Tris-HCl, pH 8.0. An elution profile was obtained on the chart recorder.

2.4. SDS-PAGE

SDS-PAGE was performed with a PROTEAN II

electrophoresis apparatus (Bio-Rad, Mississauga, Ont., Canada) and a Laemmli buffer system [23]. The separating gel contained 12% (w/v) acrylamide and 0.23% bisacrylamide before polymerization. The protein sample was mixed with sample buffer containing a final concentration of 20% (w/v) glycerol, 1% (w/v) SDS, 0.05% (w/v) bromophenol blue and 0.625 M Tris-HCl buffer (pH 6.8) and 0.025% β-mercaptoethanol. Broad range protein standard markers were used for the calibration of molecular masses. Electrophoresis was performed at 20 mA per 0.75 mm gel for approximately 1.5 h. Protein bands were visualized by the silver staining method as described by Merril et al. [24].

2.5. Metal displacement assay

To check the specificity of the purified protein for zinc, a metal displacement assay was performed by the method of Neilson et al. [25]. Scatchard plot analysis was carried out as described by Weder et al. [26]. Native protein (10 μg) reconstituted with 10 μCi $^{65}Zn^{2+}$ for 4 h was incubated at 4°C in the presence of increasing concentrations of different metal ions (Zn^{2+}, Ca^{2+}, Cu^{2+} and Cd^{2+}). After 24 h 1.0 ml of a slurry of chelax 100 (1 g/10.0 ml double distilled H_2O) was added to each sample. Supernatants from the centrifuged samples were analyzed for radioactivity (CPM).

2.6. UV absorption assay

The specificity of the protein for zinc was checked by UV absorption study as described earlier [25]. Samples of native protein (10 μg) were incubated at 4°C with different metal ions (Zn^{2+}, Ca^{2+}, Cu^{2+} and Cd^{2+}) and UV absorption spectra were recorded on Hitachi 2000 UV-vis spectrophotometer.

2.7. Carbohydrate content estimation

The carbohydrate content was checked by phenol-sulfuric acid microassay [27]. In brief, 10 μg equivalent of protein sample was incubated with 20 μl of reagent A (5% w/v of distilled phenol). 100 μl of reagent B (concentrated H_2SO_4) was added rapidly to the reaction mixture. The reaction mixture was left undisturbed for 10 min and then shaken vigorously and incubated at 37°C for 30 min. Optical density was measured at 490 nm. Glucose (1–10 μg) was used as standard.

2.8. Immunohistochemical localization

Antiserum against the purified major zinc binding protein was raised in rabbit (New Zealand White strain) and titer of antibodies was checked by enzyme-linked immunosorbent assay (ELISA) [28]. Immunofluorescence staining was performed by the method of Nartey et al. [29]. Kidney was removed from the anesthetized rat and stored at −80°C prior to cutting 10 μm sections in the cryotome. The sections were kept at −80°C for fixing. The sections were air dried and antiserum diluted (1:10) with phosphate buffered saline (PBS) was layered on the tissue sections. After incubating in a humidified chamber at 37°C for 30 min, the antiserum was removed and sections were washed several times with PBS. The FITC conjugated anti-rabbit goat IgG (1:50 in PBS) was layered on the tissue sections and incubated in a humidified chamber at 37°C for 45 min. Then sections were washed with PBS several times, mounted in 10% glycerol (v/v in PBS) and viewed under the fluorescence microscope.

3. Statistical analysis

The data are expressed as means ± S.D. of at least nine measurements on three or more separate BBM preparations.

4. Results

4.1. Purification of major zinc binding protein

4.1.1. Solubilization of BBM proteins

BBM proteins were solubilized by using *n*-octylglucoside at a concentration of 1% (w/v). The detergent treatment solubilized 80.54 ± 5.4% of the protein from BBM.

4.1.2. Chromatographic separation of solubilized proteins

The solubilized BBM proteins were subjected to

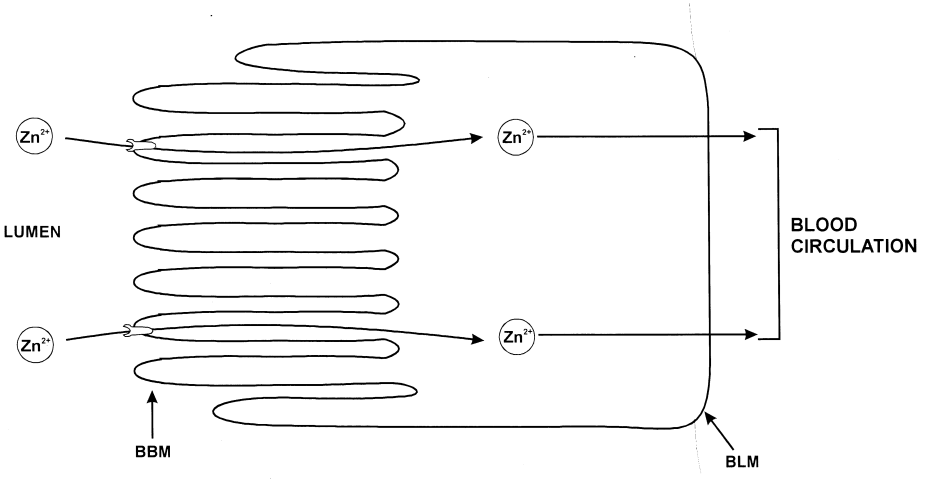

Fig. 1. Renal epithelial cell. The first barrier in the transport of solutes is the brush border membrane.

chromatographic separation procedure. DEAE-Sephadex chromatography of solubilized BBM protein yielded four peaks (Fig. 2). Peak III showed high zinc binding activity. Fractions showing high zinc binding activity were pooled and loaded onto a Sephadex G100 column. Proteins were resolved into two major peaks (Fig. 3). Peak II showed high zinc binding activity. Proteins purified on the Sephadex G100 column were loaded onto a phenyl-Sepharose column where the proteins were resolved into three major peaks (Fig. 4). Peak II showed high zinc binding activity. Fractions corresponding to this peak were pooled and purity was checked by FPLC. The protein sample showed a single peak indicating that

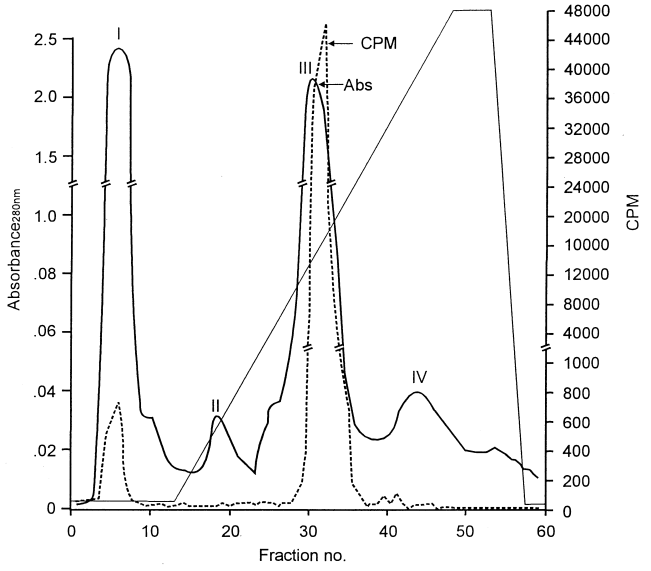

Fig. 2. Elution profile on a DEAE-Sephadex column. The solubilized BBM proteins were loaded onto a DEAE-Sephadex column, pre-equilibrated with 25 mM Tris buffer (pH 7.0) containing 2% lubrol px. Bound proteins were eluted with a linear gradient of 1 M NaCl in equilibration buffer at a flow rate of 12 ml/h.

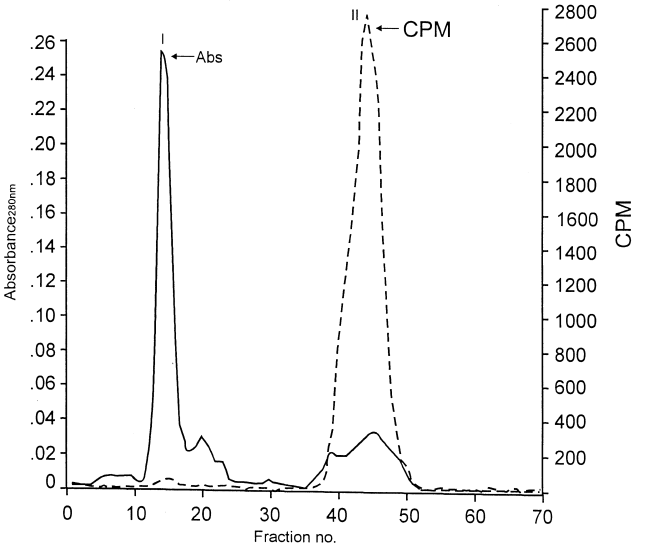

Fig. 3. Elution profile on Sephadex G100 column. The protein purified on DEAE-Sephadex was loaded onto a Sephadex G100 column equilibrated with 25 mM Tris buffer (pH 7.0) containing 2% lubrol px. The protein was eluted with the same buffer at a flow rate of 8.0 ml/h.

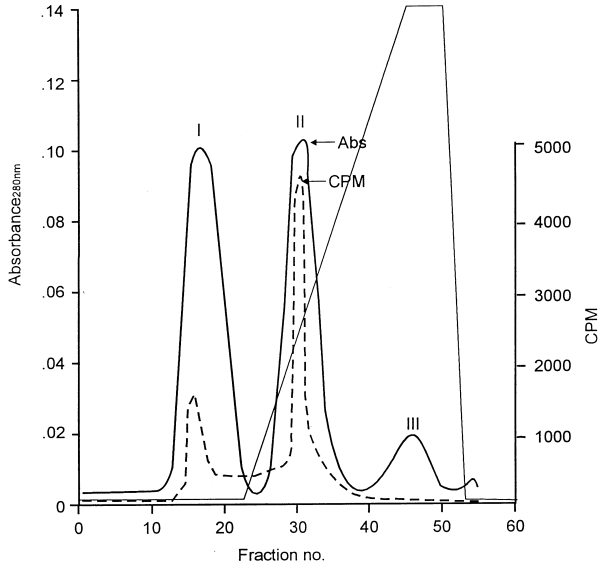

Fig. 4. Elution profile on phenyl-Sepharose 4B column. The concentrated peak II of the previous column (gel filtration) was loaded on to the phenyl-Sepharose 4B column, pre-equilibrated with 25 mM Tris buffer (pH 7.0). Bound proteins were eluted with a linear gradient of 2% *n*-octylglucoside.

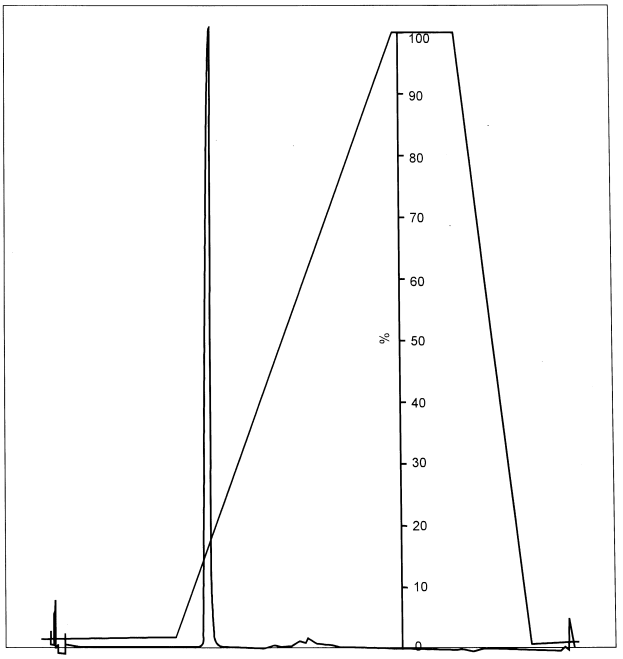

Fig. 5. Rechromatography of purified majaor zinc binding protein on FPLC. The protein purified on a phenyl-Sepharose column was loaded onto a Mono Q column preequilibrated with 10 mM Tris-HCl, pH 8.0. Bound protein was eluted with a linear gradient of 1 M NaCl at a flow rate of 1 ml/min. Elution profile was obtained on a chart recorder.

the protein had been purified up to homogeneity (Fig. 5).

Table 1 shows the purification fold and % yield of major zinc binding protein at different steps of purification. Starting from 46.75 mg of BBM protein, after solubilization, dialysis and concentration, 21.98 mg of protein was obtained with specific activity of 5836 CPM/mg protein. This activity was taken as 100%. After ion exchange chromatography 2.39 mg of protein with a specific activity of 51 924 CPM/mg protein was recovered achieving 8.89-fold purification. 15.36-fold purification was achieved after gel filtration chromatography, which yielded 190 μg of protein. This protein when resolved on a phenyl sepharose column yielded 80 μg of protein (specific activity 129 100 CPM/mg protein) achieving 22.12-fold purification. The purification was done several times to yield a sufficient amount of protein to carry out further experiments.

4.2. SDS-PAGE

Purity and homogeneity of the finally purified major zinc binding protein were further checked by SDS-PAGE (Fig. 6). The purified protein showed a single band when stained with silver nitrate and moved with apparent electrophoretic mobility of 40 kDa on a 12% gel.

Table 1
Purification fold of zinc binding protein at different steps of purification

S. No.	Purification step	Protein	Activity (CPM)	Specific activity (CPM/mg protein)	% yield	Fold purification
I	BBM	46.75 mg	–	–	–	–
II	Solubilized proteins	21.98 mg	128 275	5 836	100	–
III	DEAE-Sephadex	2.39 mg	124 098	51 924	10.87	8.89
IV	Sephadex-G100	197 μg	17 670	89 695	0.896	15.36
V	Phenyl-Sepharose	80 μg	10 328	129 100	0.364	22.12

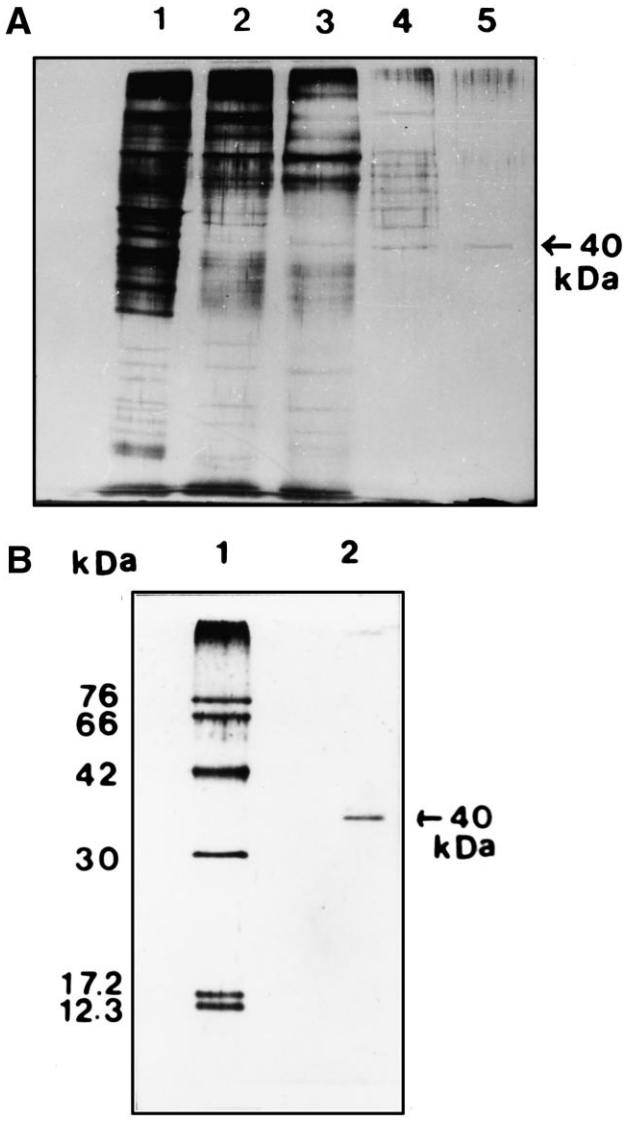

Fig. 6. SDS-PAGE of purified zinc binding protein. (A) Lane 1, BBM protein; lane 2, solubilized BBM protein; lane 3, DEAE-Sephadex purified protein; lane 4, Sephadex G100 purified proteins; lane 5, phenyl-Sepharose purified protein. (B) Lane 1, standard molecular weight markers; lane 2, phenyl-Sepharose purified zinc binding protein. The gels were stained with silver nitrate.

4.3. Metal displacement assay

The specificity of the purified protein for zinc was checked by the metal displacement assay. The protein was radiolabelled by incubating it with $^{65}Zn^{2+}$. The displacement of the bound zinc was monitored in the presence of increasing concentrations of different metals (Fig. 7A). The chelax procedure itself does not affect the recovery of metal bound protein in the supernatant. The bound zinc was displaced by the increasing concentration of Zn^{2+} and Cd^{2+} (0.5–5.0 mM) whereas even up to 5.0 mM of Ca^{2+} or Cu^{2+} had little or no effect on the displacement of zinc bound to the protein. To determine K_d and B_{max}, Scatchard plot analysis was carried out. The data were plotted as bound Zn^{2+} (B_0) on the x-axis and ratio of bound Zn^{2+} to free Zn^{2+} (B_0/B_f) on the y-

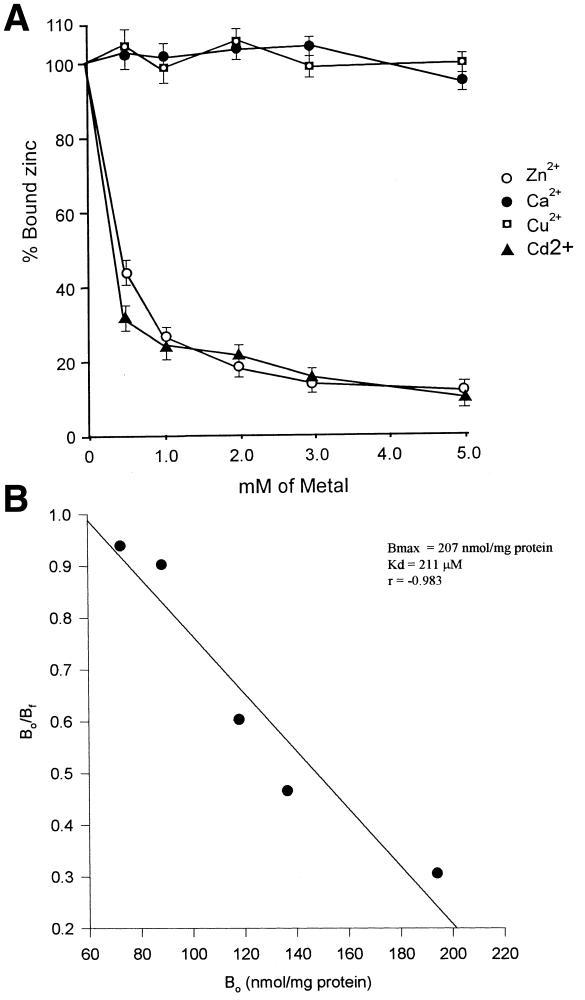

Fig. 7. Metal displacement assay. (A) 10 µg of native protein, labelled with $^{65}Zn^{2+}$, was incubated at 4°C with increasing concentrations of Zn^{2+} (○), Cu^{2+} (□), Cd^{2+} (▲) or Ca^{2+} (●). After 24 h 1 ml of chelax resin was added, centrifuged and radioactivity was counted in the supernatant. (B) Scatchard plot analysis of zinc binding sites. The concentration of bound (B_0) and free (B_f) Zn^{2+} was calculated from the above data. Best fit line was drawn by linear regression analysis. Kinetic parameters were calculated from intercept and slope of line.

axis and the best fit line was drawn by linear regression analysis (Fig. 7B) as described by Weder et al. [26]. On interpreting the data of zinc binding, one line with one slope was obtained indicating the presence of one type of binding sites for zinc. The zinc binding sites showed a K_d of 211 μM and a B_{max} of 207 nmol/mg protein. 8.0 g zinc atoms could interact per mol of protein.

4.4. UV absorption assay

The specificity of the protein for zinc was further checked by UV absorption study. The procedure involved titration of the apo-protein with increasing mol equivalents of different metals (Fig. 8). It was found that Zn^{2+} in a concentration of 0.4 mM resulted in a shift of the absorption maximum from 276 nm to 217 nm. Increasing the concentration of Zn^{2+} to 3.0 mM resulted in an increase in absorbance at 216 nm and the absorption maximum at 276 nm disappeared at 2.0 mM of Zn^{2+}. Similarly 0.5 mM Cd^{2+} completely diminished the absorption maximum at 278 nm and shifted it to 217.0 nm.

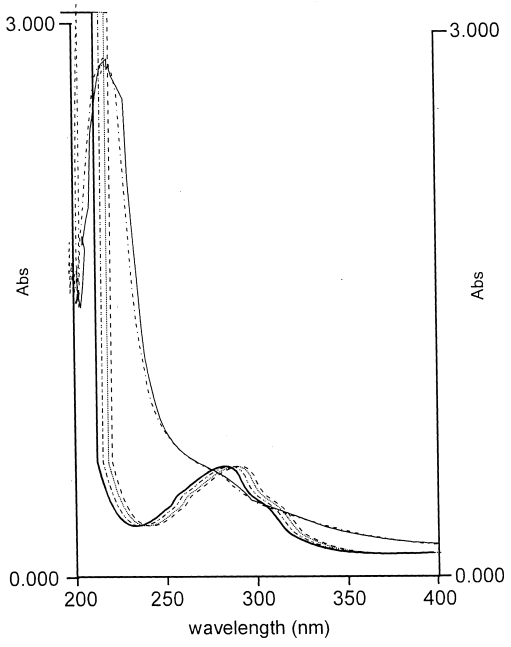

Fig. 8. UV-absorption assay. 10 μg of apo-protein was reconstituted without (————) or with 3 mM Zn^{2+} (-.-.-.-), 3 mM Cd^{2+} (————), 5 mM Cu^{2+} (—), 5 mM Ca^{2+} (...) or 5 mM Mg^{2+} (-..-..-), then absorption spectra were recorded at 200–400 nm.

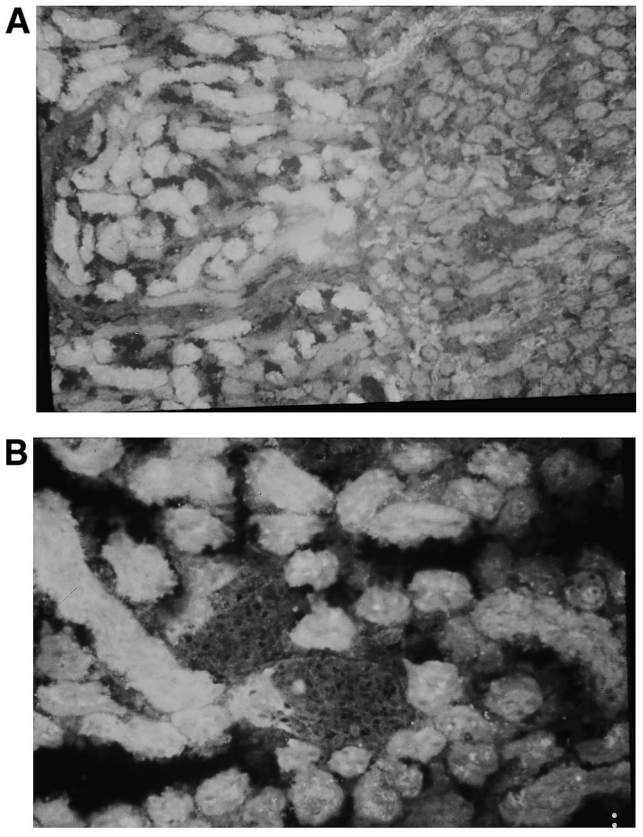

Fig. 9. Immunofluorescence localization of major zinc binding protein in rat kidney. Bright yellow fluorescence was seen in the renal cortex (A: ×100). Further at higher magnification (×400), it was found that only proximal convoluted tubules showed bright yellow fluorescence (B).

Lowering the pH to 2.5 resulted in the reappearance of the absorption maximum at 278.04 nm in both cases. However, up to 5.0 mM Ca^{2+} or 5.0 mM Cu^{2+} or 5.0 mM Mg^{2+} did not have any effect on the absorption spectra of the apo-protein.

4.5. Carbohydrate content

The glycoprotein nature of the purified protein was checked by assaying carbohydrate content of the protein. The carbohydrate content was found to be 7.58% (w/w) in the purified protein.

4.6. Immunohistochemical localization

Ultrastructural localization of major zinc binding protein in the kidney sections was carried out by immunofluorescence staining. Bright yellow fluores-

cence was seen in the cortex of the kidney (Fig. 9A). Further diffused or nonspecific fluorescence was also seen in the medulla portion of the kidney. Glomeruli did not show any reaction where as proximal convoluted tubules showed very bright yellow fluorescence (Fig. 9B). However, no fluorescence was seen in the distal convoluted tubules.

5. Discussion

Most of the studies, apart from experiments regarding ZnT-1 and ZnT-3, have been performed in either isolated cell cultures or in heterologous cell systems. Evidence for a role of any zinc transport (ZnT) protein in the actual transport of Zn^{2+} across the lipid bilayer is still wanting, to understand the molecular basis of this process. Since no protein has been identified so far which could be involved in the movement of zinc across the BBM, the first barrier in the transepithelial movement of zinc, we have made an attempt to identify, purify and partially characterize the ZnT protein from the BBM, which could be involved in this process as it occurs in the renal tubular system.

Utilizing the zinc binding property of the protein and membrane proteins solubilization procedure, we have identified and purified a 40 kDa major zinc binding protein by using different chromatographic separation columns. Purity of the major zinc binding protein was checked by SDS-PAGE upon silver nitrate staining and FPLC, which indicated that the protein had been purified to homogeneity. ZnT-Proteins cloned so far are 40–50 kDa [14–17]. Other zinc containing proteins in BBM are the enzymes, e.g. alkaline phosphatase (160 kDa) and leucine aminopeptidase (255 kDa). These enzymes are of high molecular weight. Therefore it can be speculated that the 40 kDa protein purified from the BBM might be involved in the transport of zinc across the BBM in the renal tubules.

The functional stability of the protein after solubilization and chromatographic separation procedure was checked by metal displacement assay. Further, specificity of the protein for zinc was also investigated by monitoring the displacement of bound zinc in the presence of different metal ions. It was found that Zn^{2+} and Cd^{2+} could displace the $^{65}Zn^{2+}$ bound to the protein. In vitro studies using BBM vesicles have also shown that Cd^{2+} competitively inhibited the Zn^{2+} uptake process in these vesicles [11,12]. The inhibition of zinc uptake by Cd^{2+} has also been observed in other mammalian systems where carrier mediated uptake of zinc has been demonstrated in liver parenchymal cells [3], intestine [10], kidney [11], human lymphocytes and fibroblasts [30,31]. However, Cu^{2+}, Ca^{2+} and Mg^{2+} could not displace the protein bound Zn^{2+}. These findings suggested that the purified protein is specific for Zn^{2+} only and Cd^{2+} competes for the same types of binding sites on the protein. Scatchard plot analysis revealed the presence of only one type of zinc binding sites with high K_d and B_{max}, indicating that these binding sites have high affinity and capacity for zinc.

The specificity of the purified protein for Zn^{2+} was further confirmed by UV absorption assay. Shifts in absorption maxima of the proteins in response to binding of metals are well documented [25]. The binding of Zn^{2+} or Cd^{2+} resulted in a shift of the absorption maximum to a lower wavelength, indicating that both metal ions are binding to the same binding sites. It is well reported in the literature that renaturation of apo-metallothionein with Cd^{2+} and Zn^{2+} resulted in a shift in the absorption maximum [32,33]. A characteristic feature of the metalloproteins is their prominent absorption spectra which result from metal-ligand charge transfer transitions. The shape and extinction coefficients of the spectrum are distinctive for different metals bound to the protein [34–37]. Increasing the metal equivalency in subsaturating amounts in metallothionein samples resulted in an increase in the absorbance [38]. Various metal ions were screened for their ability to enhance the UV absorption of apo-protein. Only Zn^{2+} and Cd^{2+} resulted in a significant increase in UV absorption as a function of added metal at shifted absorption maxima. Even 5.0 mM of Ca^{2+} or Mg^{2+} or Cu^{2+} had no effect on the characteristic absorption spectra of the protein. These results further substantiated the finding that the purified protein is specific for Zn^{2+}. The carbohydrate content was found to be 7.58% (w/w), suggesting that the purified protein is glycoprotein in nature.

Immunohistochemical localization of purified major zinc binding protein in kidney revealed that this protein is mainly localized in the proximal convo-

luted tubules. No fluorescence was seen in renal medulla and the distal convoluted tubules. Proximal convoluted tubules are the active site of reabsorption of most of the metal ions. Therefore, it can be speculated that this protein might be involved in the reabsorption of zinc across the BBM in the renal proximal convoluted tubules.

The 40 kDa purified protein is the first protein to be identified and purified from renal BBM which has high specificity, affinity and binding capacity for Zn^{2+}. This protein is glycoprotein in nature and might be involved in the uptake of zinc into the epithelium of the renal proximal tubules. To establish the role of this purified protein in zinc transport, further studies are in progress to investigate its transport characteristics after reconstituting it into the proteoliposomes.

Acknowledgements

We thank Assistant Professor Dr. Ranjana Minz, Department of Immunopathology, PGIMER, Chandigarh, India, for her kind help during the immunohistochemical localization study. This project was financed by the Council of Scientific and Industrial Research, New Delhi, India (Sanction Number 37(0957)/97/EMR-II).

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